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THESIS OF DOCTORAL (PhD) DISSERTATION

EFFECT OF METRITIS ON THE REPRODUCTIVE PERFORMANCE OF DAIRY COWS

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I. ANTECEDENTS OF THE RESEARCH

The bacterial complications of uterine involution and the different kinds of metritis, i.e. *puerperal metritis*, *clinical endometritis, pyometra* and *subclinical endometritis* (SHELDON, I.M., LEWIS, G., LEBLANC, S., GILBERT, R. (2006): Defining postpartum uterine disease in dairy cattle. Theriogenology, 65. 1516-1530.) are regarded as the most common forms of genital diseases in dairy cows. These pathological conditions may delay the complete histological regeneration of the endometrium and disrupt the resumption of cyclic ovarian function, resulting in postponement of the first postpartum (pp) insemination (AI), increasing the number of AIs per conception, and thus prolonging the calving interval and decreasing the calving rate (HUSSAIN, A.M., DANIEL, R.C.W. (1991a): Bovine normal and abnormal reproductive and endocrine functions in the postpartum period: a review. Reprod. Dom. Anim., 26. 101-111.).

As for the dairy farms the most important source of income is the sale of milk, and since milk production of cows can be expected only after calving, it is extremely important to ensure that our cows should conceive without delay. The economic efficiency of cattle production is basically determined by the calving interval, i.e. the length of time that elapses between two consecutive parturitions. The calving interval greatly depends on the service period (number of days open, i.e. the calving-to-conception interval). The length of the service period is a good indicator of the involution conditions and reproductive status of the given dairy farm.

The opinions of both veterinarians and animal breeders vary as to the optimum length and economic impact of the calving interval. However, we can state that the optimum length of the calving interval always depends on the herd studied, and primarily on its production.

Monitoring of the process of uterine involution as well as early diagnosis and treatment of the possible complications makes it possible to achieve the soonest possible re-conception of cows and, thus, to decrease the number of days open and shorten the calving interval. Taken together, these outcomes provide substantial benefit for the dairy farms and, at the same time, justify the studies conducted by us.

II. AIMS OF THE RESEARCH

We studied puerperal metritis (PM) in a complex series of trials **(Trial 1)**. On the one hand, we studied the effect of (supposed) predisposing factors (manual intervention during calving, retained placenta, energy-deficient status of the cow) on the incidence of the disease. Are there any other factors influencing the occurrence and severity of PM? On the other hand, what are the important clinical features of PM (appearance of the pathognomic sign, occurrence of fever, reduced milk production, nutritional status) and is there a correlation with PM and other diseases of involution (mastitis, clinical endometritis, foot problems)? Furthermore, we studied the effect of PM on the milk production and reproductive performance of cows.

We determined the antibiotic susceptibility patterns of primary uterine pathogens cultured from uterine swab samples of cows showing signs indicative of complications of uterine involution (puerperal metritis, PM or clinical endometritis, EM) **(Trial 2: 2a and 2b).** Is there any alteration of the minimal inhibition concentration (MIC) to different antibiotics?

We also studied whether there is a correlation between decompensated negative energy balance (NEB) characterised by ketonuria and the course of puerperal metritis, and whether the efficiency of antimicrobial therapy used for the treatment of metritis can be improved by the simultaneous administration of antiketogenic agents and, consequently, whether the incidence of further genital diseases (clinical endometritis) can be reduced and the reproductive indices improved **(Trial 3)**.

Over several years, we conducted a series of trials in large-scale Hungarian dairy farms to assess the efficacy of different antimicrobial active ingredients used against PM **(Trial 4a)** and clinical EM, as well as (in the case of EM – **Trials 4b, 4c and 4d**) the efficacy of $PGF_{2\alpha}$ used alone or in combination with antibiotics. This evaluation was primarily based on reproductive and, partially, bacteriological effects. On one occasion (**Trial 4b**) we included in the study a group treated with an inorganic iodine product, which represents the oldest but still rather widely used method of treatment.

III. METHODS OF THE RESEARCH

Common features of the studies: the field trials were carried out altogether in 20 dairies (farm sizes were between 300 and 1,850 cows) with Holstein-Friesian (HF) or HF and Hungarian Fleckvieh crossbreed cows, over several years, in general from late autumn up to the end of spring seasons. Management and feeding conditions represented the average circumstances in Hungary (HUSZENICZA, GY., FODOR, M., GACS, M., KULCSAR, M., DOHMEN, M. J. V., VAMOS, M., PORKOLAB, L., KEGL, T., BARTYIK, J., LOHUIS, J. A. C. M., JANOSI, SZ., SZITA, G. (1999): Uterine bacteriology, resumption of cyclic ovarian activity and fertility in postpartum cows kept in large-scale dairy herds. Reprod. Dom. Anim., 34. 237-245.). Neither grazing nor regular moving was not possible for the animals. Calving was performed in continuously used calving barns under supervision of trained and skilled staff. At admission to the trials, a substantial genital tract injury, vaginal or uterine prolepses, and previous systemic or intrauterine (iu.) antibiotic treatments were exclusion criteria.

PM was defined as the complication of early puerperium (usually between days 5-10 pp up to maximum of 14 days pp) which was sometimes accompanied by severe general signs. Local symptoms were: malodorous, watery reddish-brown uterine discharge, atonic, thin or oedematous thick uterine wall.

EM was defined as the complication of middle or late involution period. The key parameter to the diagnosis was the quality of uterine discharge: mucopurulent (pus content <50%); purulent (pus content >50%); or foul smelling, blood containing discharge was the criteria. Diagnosis was established by rectal and vaginoscopic examinations in both diseases.

At the statistical hypothesis tests between groups comparison of *continuous variables* in the case of approximately normal distribution was performed by one-way analysis of variance (**ANOVA**). When ANOVA provides significant difference between groups by means of F value and degree of freedom (df), the pair wise comparisons were made by the least significant difference method (LSD) and the LSD value at p=0.05 level was given ($LSD_{P=0.05}$). If the conditions of ANOVA (closely normal distribution and similar variance) were not satisfied, **Median test** was performed (e.g. length of service period). For discrete variables **Chi-square** or **Fisher's exact** test was performed. The particular and combined effect of predisposing factors was studied by **logistics regression model**. Odds ratios (OR) and their 95% confidence intervals (CI) were calculated by the model. A combined comparison of fertility rate and length of service period was performed by **cumulative survival function** when curves were compared by **Mantel-Haenszel test** (DINYA, E. (2001): Biometria az orvosi gyakorlatban. Medicina Kiadó, Budapest, 291-317; 383-393.).

In **Trial 1,** we studied puerperal metritis (PM) under large-scale farm conditions in two Hungarian dairies, in secundiparous or older Holstein-Friesian cows (n=170).

On both farms, we selected those cows calving between 1 February and 30 April and starting at least their second lactation which (1) required manual calving assistance during calving, or (2) retained placenta occurred, or (3) ketonuria was detected 24–48 hours after calving. In addition, we included in the trial herdmates of the above cows, selected by the method of fitted pairs that (1) had calved within 14 days, (2) were similar in terms of milk production in the previous lactation as well as in terms of parity/age, (3) were not affected by any of the problems listed above. During clinical examination performed 24–48 hours after calving we checked whether the placenta had been passed, the body condition was scored, and urine acetoacetate (AcAc) as well as blood BHB levels were determined. Between postpartum days 3 and 15, we took the rectal temperature at 24-hour intervals and recorded the cows' milk production, occurrence of PM and mastitis on daily basis. Within the group affected by PM, we distinguished a severe form designated as toxic PM (tPM) which manifested itself, in addition to the local signs, in systemic (septic) signs such as depression, inappetence and pyrexia. Clinical examination carried out between pp days 28 and 35 aimed the investigation of clinical EM and repeated evaluation of the body condition. Subsequently, reproductive and clinical data were collected up to postpartum day 150.

In **Trial 2** we carried out two series of experiments. During the first one (1998) uterine swab samples were taken on pp days 10, 20 and 28–35 from cows having PM or clinical EM. At the second one (2004) samples were taken from cows affected with PM when the diagnosis of the disease was established.

A sterile, double-sheathed disposable uterine swab (IMV®, France) was used. The swab was transported in a transport medium (Port-A-Germ®) which ensured survival of the entire bacterial flora, including also the sensitive Gram-negative anaerobes (GNA). The samples were processed in the laboratory within 24 hours. For the isolation of bacteria, the swabs were surface-streaked onto general and selective solid media (blood agar, chocolate agar, eosin-methylene blue agar, as well as AVA and SAVA), and then the inoculated plates were incubated under aerobic and anaerobic conditions. Aerobic bacterial strains were identified using the ATB automatic identification system of Bio-Mérieux. For the identification of anaerobic strains we used the Bio-Medica REMEL ANA ID panel II semi-automatic system.

In 1998, we determined the MIC values (µg/ml) of 7 antibiotics [amoxicillin, cephapirin, oxytetracycline (OTC), gentamicin, neomycin, norfloxacin and tylosin] against *Arcanobacterium (A.) pyogenes* and *Escherichia (E.) coli* strains isolated from cases of PM and clinical EM, using the agar dilution procedure (nutrient medium: *Isosensitest agar®, Oxoid Ltd, UK;* we prepared a doubling dilution series, the concentration of the antibiotics tested was 0.032–64.0 µg/ml). In 2004, the MIC was measured on microtitre plates. The susceptibility of *A. pyogenes, E. coli* and four groups of Gram-negative anaerobe strains isolated from PM cases to four antibiotics (OTC, amoxicillin, cephapirin and cefquinom) was determined. The MIC was regarded as the lowest antibiotic concentration at which bacterial growth was no longer observed. The MIC_{50} (50% of the strains did not grow) and the MIC_{90} (90% of the strains did not grow) values were also determined.

In **Trial 3** we studied the correlations between ketonuric state and the course of puerperal metritis (PM) on two dairy farms. The trial included all cows of the two dairy herds that calved at term (>270 days) and fell ill within the first 10 days post partum, showing the pathognomonic signs of PM. After having established the diagnosis, we used intrauterine (iu.) antimicrobial therapy supplemented with the administration of uterotonics or prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). This treatment was repeated every 24–48 hours until the vaginal discharge lost its putrid character. If clinical EM had developed medication was continued ($PGF_{2\alpha}$ products, or uterine washing with 1% Betadine solution, or antibiotic-containing uterine infusion until the cervical mucus became clear and transparent. We recorded the number of treatments and the length of the interval between calving and the last treatment. Anatomical involution of the uterus was monitored by rectal and vaginal examination performed twice a week. To determine the time of re-conception and the proportion of cows that re-conceived, we collected reproductive data until postpartum day 150.

Before the first uterine treatment we collected urine samples with a catheter to determine the presence of ketonuria (the quantity of AcAc excreted in the urine). At the time of starting antimicrobial therapy (day 1), a certain proportion of the animals, grouped randomly, received intravenous and oral antiketogenic treatment (Ketogenin infusion® and Ketogenin powder®), while the remaining cows did not receive such treatment. Further antiketogenic treatment of the ketonuric group depended the AcAc level: Ketogenin powder® for all animals and beside that Ketogenin infusion® for those ones with >4 mmol/l AcAc level. Thus, from the point of view of evaluating the results, a ketonuric and a non-ketonuric group was obtained, each of the two including a group having received antiketogenic treatment and another group that did not receive such treatment. From the ketonuric animals we collected urine samples daily. In the ketonuric – treated group antiketogenic therapy was continued until ketonuria was no longer detectable by the analysis of urine samples taken on two consecutive days. The non-ketonuric cows receiving antiketogenic treatment were given only oral antiketogenic treatment on days 2 and 3.

In **Trial 4** animals were enrolled at typical occurrence of PM i.e. 5-10 days pp (n=118) or clinical EM i.e. 28-35 days pp (n=871). Clinical and fertility data were collected up to the time of re-conception after calving or up to the time of culling.

Samples for bacteriological examination were taken on postpartum days 28–35 in this trial. The objective of this was to check the post-treatment bacteriological status in cows with PM and to assess the pretreatment bacteriological status in cows with EM. Swab samples were taken and processed as described before.

During **Trial 4a,** cows exhibiting PM (n=118) were divided into three groups, and treated with products containing oxytetracycline (OTC), amoxicillin (AMO) or gentamicin (GEN). Topical (intrauterine, IU) treatment was administered to all cows, while systemic treatment was applied to those ones having systemic signs of the disease with the same active component. Iu. treatments were repeated in 1-3 days apart depending on the manufacturer's recommendations, until clinical cure.

The clinical EM treatment studies were conducted on a total of 871 cows (**Trial 4b:** n=317; **Trial 4c:** n=278; **Trial 4d:** n=276). Uterine swab samples were taken at admission for bacteriological examination (first of all to prove the existence of *A. pyogenes* infection). Subsequently we assigned the cows to the following treatment groups:

4.b: untreated control; $PGF_{2\alpha}$ (im.); cephapirin (iu.); penicillin+neomycin (iu.); Iodine (iu.)

4.c: untreated control; $PGF_{2\alpha}$ (im.); cephapirin (iu.); cephapirin (iu.) + $PGF_{2\alpha}$ (im.)

4.d: untreated control; $PGF_{2\alpha}$ (im.); tilosin (im.); tilosin (im.) + $PGF_{2\alpha}$ (im.)

When evaluating the efficacy of PM treatments (**Trial 4a**), the major variables were primarily the fertility rate, in the case of cows that conceived the length of time that elapsed until conception, as well as the frequency of isolation of primary uterine pathogens (*A. pyogenes, E. coli* and Gram-negative anaerobes) from bacteriological samples taken in week 5. As complementary variables, the duration of PM treatments, the number of necessary treatments, the ratio of parenteral treatments, and the incidence of EM in postpartum week 5 were used in the comparison of treatment groups.

The efficacy of different treatments of EM (**Trials 4b–4c–4d**) was evaluated on the basis of the fertility (conception) rates. As a first step, we analysed the data of cows in the treatment groups independently of the pretreatment bacteriological status. Subsequently, we compared the data of cows that proved to be infected by *A. pyogenes* at the pretreatment bacteriological examination also separately.

IV. PRINCIPAL STATEMENTS OF THE DISSERTATION

Trial 1

During the statistical analyses, we separately evaluated the effect of different predisposing factors on the development and severity of puerperal metritis. In the case of manual intervention (assistance) during calving and retained placenta significantly ($P<0.001$) more cases of PM developed; however, these factors did not have an effect on the severity of the disease. Studying the influence exerted by energy-deficient status of the cow we demonstrated that higher BHB levels predispose cows to the development of metritis and increase the probability that the severe form of metritis will develop. When BHB level was taken as a continuous variable, the odds ratio of PM was 6.97 (CI 2.29–21.14) considering all the animals, while the odds ratio of tPM was 11.47 (CI 1.62–312.13) for all cows with PM. A ketonuric reaction of at least 2+ predisposes cows to metritis, while a more strongly positive test increases the likelihood that the more severe form of metritis will develop. By performing logistic regression we found that the elevation of the BHB level significantly increases the incidence of PM ($P=0.0004$) and, among cows that are already suffering from PM, also the incidence of tPM ($P=0.0057$).

Using logistic regression, we studied the combined effect of predisposing factors as well. As a first step, the model was constructed by including exclusively the above-mentioned and separately studied predisposing factors. First we performed a correlation test to elucidate the independence of the explanatory variables. According to the result of that test, there was a very close correlation between the BHB level and ketonuria ($r=0.84$). This means that both explanatory variables cannot be incorporated into the same regression model. From the two variables we arbitrarily chose the BHB level. In the first model constructed in this way all three variables had a strongly significant effect on the development of PM ($p<0.0001$). However, only the BHB level and the occurrence of retained placenta, and not the manual intervention carried out during calving, exerted a significant effect on whether tPM, i.e. the more severe form of PM, occurred within PM. However, the explanatory force of this model is rather low: 31.76% for PM and 28.66% for tPM.

Therefore, we tried to find further possible influencing factors among the data available to us. Taking each factor separately, we studied the effect of the following factors on the incidence of PM and tMP: milk production in the previous lactation, change in the quantity of milk produced on postpartum days 3–5, parity, body temperature, and change of the body temperature.

Average milk production in the previous lactation by group was as follows: mild PM (mPM): 8700±753 litres (n=41); tPM: 9899±1019 litres (n=15); healthy: 8731±1221 litres (n=114). Milk production in the previous lactation was >1000 litres higher in the tPM group than in the other two groups. Studying the cumulative distribution of milk production in the previous lactation in the three groups, it was found that more than 80% of the cows with tPM produced more than 9400 litres of milk in the previous lactation, while in the other two groups barely 30% of the cows belonged to that category. Studying milk production and its change on postpartum days 3 and 5, in the healthy and the mPM groups the milk yield increased between postpartum days 3 and 5 (average change: healthy: +1.78 litres, mPM: +1.49 litres), while in the tPM group it decreased (average change: –3.48 litres). According to the results of evaluation by logistic regression, the decrease of milk production showed a significant correlation with the increasing incidence of PM (P=0.0023) and tPM (P=0.0091). The parity number does not exert a significant effect on the development of PM but it significantly affects its severity (P=0.058). Analysing the data of body temperature taken on both postpartum days 3 and day 5 on all animals by Fisher's exact test it was found that the ratio of cows with low and high body temperature significantly differed (P<0.0001) in the mPM, toxic PM and healthy groups (P<0.0001). At the same time, evaluating only the cows suffering from metritis, the only on postpartum day 3 did the distribution of mPM and tPM in cows with low and high body temperature differ significantly (P=0.0367). Logistic regression analysis of daily body temperature data, the higher temperature at both postpartum day 3 and 5 significantly shows the occurrence of PM and tPM, while analysing the temperature change between pp day 3 and 5; elevation proved to be a significant marker of PM (P<0.0001) and tPM (P=0.0074).

The odds ratios of the factors analysed for the development of PM, taking into account all cows included in the study, are presented in **Table 1**, while their odds ratios for the development of tPM, taking into account only the cows that already suffer from metritis, are shown in **Table 2**.

Table 1. Odds ratios of the factors analysed for the development of PM

Factor	Odds ratio	CI
Incidence of any type of PM among all cows studied		
Manual intervention during calving	6.66	2.54 - 17.51
Retained placenta	13.50	5.01 - 36.36
BHB >1.2 mmol/l	6.13	2.88 - 13.02
Ketonuria ++	3.71	1.65 -8.36
Ketonuria score of at least +++	4.03	1.49 - 10.91
Milk drop on postpartum days 3–5	9.14	3.73 - 22.42
Body temperature >39.5 ºC on day 3	12.33	3.32 - 45.88
Body temperature >39.5 ºC on day 5	8.5	3.8 – 19.02
Body temperature elevation on days 3–5	16.27	5.91 - 44.76

Table 2. Odds ratios the factors analysed for the development of tPM among cows with metritis

Factors	Odds ratio	CI
Incidence of tPM among the cases of PM		
Manual intervention during calving	2.71	-
Retained placenta	3.86	1.05 - 14.22
BHB >1.2 mmol/l	6.25	1.44 - 27.21
Ketonuria ++	3.08 ns	0.58 - 16.26
Ketonuria score of at least +++	14.0	2.3 - 85.26
Milk production > 9400 litres	23.11	4.09 - 130.56
Milk production drop on days 3–5	6.67	1.44 - 30.94
Parity number	3.14	1.36 - 7.29
Body temperature >39.5 ºC on day 3	4.25	1.72 - 11.44
Body temperature >39.5 ºC on day 5	3.88	1.0 - 15.07
Body temperature elevation on day 3–5	12.46	2.02 - 76.66

Subsequently, by incorporating the above explanatory variables we again constructed the models: two models for examining the effect of factors influencing PM and another two for examining the effect of factors influencing tPM, since BHB and ketonuria could be incorporated into the model only separately.

The following results were obtained if the BHB level was incorporated into the model:

When studying the effect of the predisposing factors on the incidence of PM, we found that the BHB value, retained placenta, manual intervention during calving significantly increases the chance of metritis and the elevation of body temperature is the marker of development of the disease.

Studying the effect of predisposing factors on the incidence of tPM among cows with metritis, we found that the BHB value, retained placenta, manual intervention during calving and high milk production in the previous lactation significantly increase the chances of development of metritis. However, the effect of BHB and that of high production do not add up.

The following results were obtained if ketonuria was incorporated into the model:

When studying the effect of predisposing factors on the incidence of PM in the model, we found that ketonuria (both the 2+ reactions and the reactions of at least 3+), retained placenta, manual intervention during calving significantly increases the chance of metritis and the elevation of body temperature is the marker of development of the disease.

When studying the effect of predisposing factors on the incidence of tPM, we found that the incidence of toxic PM among cows with metritis can be estimated on the basis of milk production in the previous lactation, the parity number, the degree of ketonuria and manual intervention carried out during calving as predictors and the decrease of milk yield between pp day 3 and 5 as first sign.

We can state that the models constructed by incorporating the predisposing factors assumed by us already earlier as well as the newly applied influencing and diagnostic factors are suitable for predicting both the incidence and the severity of metritis. The parity number and the milk production in the previous lactation are data that are available for all cows. Manual intervention (assistance) during calving and the occurrence of retained placenta are data that are available within 24 hours of calving. If we complement this with the study of a parameter (ketonuria or BHB) indicating the metabolic status of the cow during the first 24 hours post partum, we can predict the chance of development of puerperal metritis in a given cow with sufficient accuracy. The accuracy of this prediction can be increased further by systematic screening for first clinical signs i.e by taking the temperature of cows in the calving barn daily until release, and by the daily recording of the quantity of milk produced. The latter is (or would be) especially important after the cows are released into the producing group (postpartum day >5), as often the decrease of milk production is the only sign that, besides the routine clinical examination findings, calls our attention to the development of PM (or other diseases of involution). The use of this model facilitates the soonest possible diagnosis of these diseases, thus the necessary treatments can be started sooner, which is expected to result in earlier re-conception and higher re-conception rates.

In cows with toxic puerperal metritis accompanied by severe systemic signs, the pathognomonic sign, i.e. putrid vaginal discharge, can be observed already on postpartum day 3 in 40% of the affected animals, and this sign appeared in all cows by postpartum day 9. At the same time, in mild cases of puerperal metritis not accompanied by systemic signs only 29% of the cows showed this sign on postpartum day 5, and only on postpartum day 10 could the putrid discharge be observed in all animals. Evaluating the average body temperature data of the three groups by one-way analysis of variance (ANOVA), we found significant differences ($P<0.0001$), and the difference between any two out of the three groups was also significant ($P<0.0001$ in all cases). Between postpartum days 3 and 15, all but two cows affected with PM (96%) had fever on at least one day, and the same occurred in 17% of the cows without PM. The average number of days with pyrexia was 4.1 in the mPM and 7.9 in the tPM group. If the febrile condition in itself is used as the principal diagnostic criterion, a relatively large number of animals will be misdiagnosed as having PM and, what is an even bigger problem, about half of the cows actually suffering from PM will not be considered sick, unless we take the temperature of cows daily until postpartum days 14–15, which is very uncommon in the practice of dairy farms and difficult to carry out with cows in the producing group.

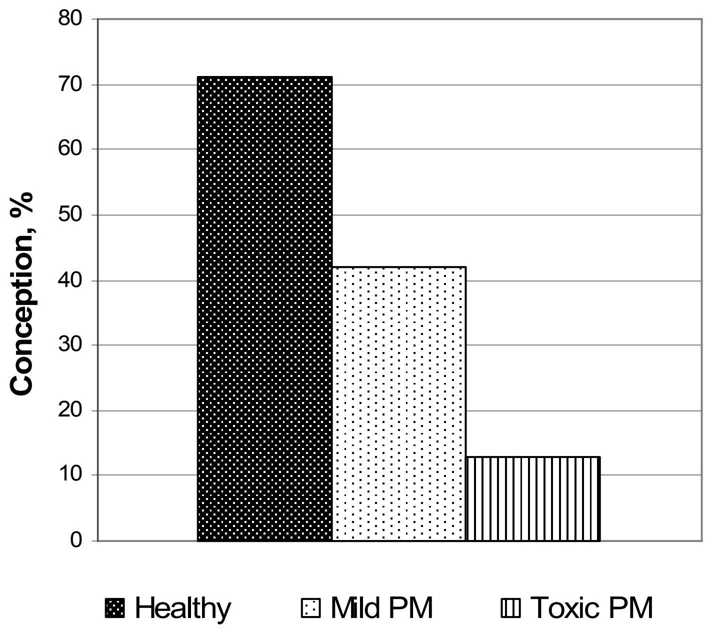
Mastitis, clinical endometritis, pododermatitis and arthritis equally occurred with a higher probability in cows suffering from metritis. The loss of body condition was significantly different ($P<0.0001$) in the healthy group and in the group suffering from toxic PM. The effect of metritis on the milk production of cows was also studied: milk production was significantly different in the three groups ($P<0.0001$), and a significant difference was found between any two out of the three groups ($P<0.0001$ in all three cases). The average milk production of cows with mPM between postpartum days 3 and 15 was 6.54 litres lower [CI: –8.12 litres; –4.97 litres] while that of cows suffering from tPM was 15.54 litres lower [–17.83 litres; –13.27 litres] than that of their healthy herdmates.

Using linear regression, we studied the change of milk production over time. In the healthy group milk production significantly increased with time (day 3: 17.3 ± 3.8 litres; day 13: 31.1 ± 4.9 litres; day 15: 35.1 ± 5.3 litres; $P<0.0001$), whereas in the mPM group it decreased (day 3: 18.4 ± 4.1 litres; day 13: 17.6 ± 4.3 litres; day 15: 20.1 ± 4.6 litres; $P=0.0385$), and in the group of cows suffering from toxic PM it also decreased significantly (day 3: 15.5 ± 6.3 litres; day 13: 7.3 ± 2.5 litres; day 15: 8.7 ± 2.9 litres;

P<0.0001). On the last two days the milk production curve already tended to rise in the affected groups (mPM and tPM), which can be attributed to the effect of the treatment started.

According to results of statistical analysis using the chi-squared test, the distribution of conceived and non-pregnant cows was significantly different in the groups suffering from metritis of different severity (mPM: 17 pregnant; 24 non-pregnant; tPM: 2 pregnant; 13 non-pregnant; P<0.001) **(Figure 1)**.

Figure 1. Ratio of cows conceiving and those failing to conceive within 150 days



According to pairwise comparison, the calving-to-conception interval was significantly different in the three groups (healthy: 103.8±23.1 days, mPM: 134.9±17.9 days, tPM: 144.0±4.2 days) (P<0.0001 in all three cases).

Trial 2

In both studies, we determined the minimal inhibitory concentrations (MIC) of antibiotics commercially available (at that time) and seeming to be suitable for topical (iu.) and/or systemic (im., iv.) treatment against aerobic uterine pathogens (*A. pyogenes* and *E. coli*). These data are presented in **Tables 3 and 4.** This allows us to follow the change of MIC values over time. From our results it can be seen that the susceptibility of *A. pyogenes* and *E. coli* strains to antibiotics traditionally and widely used for uterine treatments (OTC, amoxicillin, neomycin) had not been ideal already in 1998, and this situation deteriorated even further up to 2004, mainly with regard to *E. coli.* In contrast, cephapirin, a first-generation cephalosporin, showed equally good MIC values against *A. pyogenes* in both studies, while cefquinom, a relatively new, fourth-generation cephalosporin seems to be highly effective against both aerobic pathogens *in vitro*.

Table 3. Minimal inhibitory concentrations (MIC, µg/ml) of antibiotics against *A. pyogenes* during the two studies

	Studies conducted in 1998		Studies conducted in 2004	
	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}
Amoxicillin	4.0	8.0	0.06	0.25
Cephapirin	0.064	0.25	0.015	0.125
Oxytetracycline	8.0	32.0	0.25	32.0
Gentamicin	4.0	8.0	-	-
Neomycin	16.0	≥64.0	-	-
Norfloxacin	4.0	16.0	-	-
Tylosin	0.128	0.50	-	-
Cefquinom	-	-	0.015	0.5

Table 4. Minimal inhibitory concentrations (MIC, µg/ml) of antibiotics against *E. coli* during the two studies

	Studies conducted in 1998		Studies conducted in 2004	
	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}
Amoxicillin	0.064	0.128	4.0	64.0
Cephapirin	8.0	32.0	8.0	16.0
Oxytetracycline	1.0	≥64.0	64.0	≥128.0
Gentamicin	0.5	16.0	-	-
Neomycin	4.0	32.0	-	-
Norfloxacin	0.064	0.064	-	-
Tylosin	≥64.0	-	-	-
Cefquinom	-	-	0.06	0.275

In our studies, Gram-negative anaerobes showed adequate sensitivity to cephalosporins *in vitro*. Members of the *Bacteroides gracilis* and *Fusobacterium* group (kanamycin-sensitive Gram-negative anaerobes) were more susceptible to the antibiotics tested by us than the other Gram-negative anaerobes (**Table 5 and Table 6**).

Table 5. Minimal inhibitory concentrations (MIC, µg/ml) of antibiotics against *Prevotella bivia* and the *Porphyromonas – Prevotella* group (Gram-negative anaerobes) based upon a study conducted in 2004

	Prevotella bivia group		*Porphyromonas – Prevotella* group	
	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}
Amoxicillin	2.0	64.0	8.0	64.0
Cephapirin	0.125	≥1	≥1	≥1
Oxytetracycline	32.0	128.0	32.0	128.0
Cefquinom	≥1	≥1	≥1	≥1

Table 6. Minimal inhibitory concentrations (MIC, µg/ml) of antibiotics against the *Bacteroides fragilis* group and the *Bacteroides gracilis* and *Fusobacterium* group (Gram-negative anaerobes) based upon a study conducted in 2004

	Bacteroides fragilis group		*Bacteroides gracilis* and *Fusobacterium* group	
	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}
Amoxicillin	64.0	64.0	0.03	0.06
Cephapirin	≥1	≥1	0.015	0.015
Oxytetracycline	32.0	64.0	0.5	1.0
Cefquinom	≥1	≥1	0.03	≥1

Nowadays, the traditionally applied tetracyclines are already less effective (the MIC has increased considerably), and recently the third-generation or fourth-generation tetracyclines can be recommended most for this purpose. For the treatment of clinical endometritis, pyometra or subclinical endometritis the first-generation cephalosporins and/or $PGF_{2\alpha}$ and its analogues are the drugs of choice. By determining, for the first time, the MIC values of cephalosporins against anaerobic pathogens isolated from uterine swab samples collected in Hungary, we have found that the cephalosporins tested (cephapirin, cefquinom) are effective.

Trial 3

Studying the conditions of calving prior to selection of cows for the trial, it can be established that the incidence of retained placenta was much higher (25%) among cows found to be ketonuric than among the non-ketonuric cows (9%). Ketonuric cows not treated with antiketogenic agents excreted AcAc in their urine for 1–4 weeks, as opposed to ketonuric and treated cows in which AcAc excretion in the urine lasted for

2–8 days. In ketonuric cows not receiving antiketogenic treatment the involution of the uterus was completed about 1 week later than in the other three groups. To get rid of symptoms of uterine disease, these cows required treatment over a longer period of time and on more occasions than animals of the other three groups. In addition, these cows conceived later and in substantially lower proportions, although the difference in the length of the calving-to-re-conception interval did not reach the level of $p<0.05$ **(Table 7)**.

Table 7. Effect of ketonuria on the course of puerperal metritis and on the rate and time of re-conception

	Ketonuric, non-treated (A)	Ketonuric - treated (B)	Non-ketonuric-non-treated (C)	Non-ketonuric, treated (D)	One-way ANOVA	$LSD_{p=0.05*}$	Chi^2 test
Number of cows per group (n)	37	38	27	29	-----	-----	-----
Duration of ketonuria (in days)[1]	17.3±1.6^{§,a}	4.5±0.4^{§,b}	------	------	-----	-----	-----
Time of involution (days)[1]	38±1^{a}	31±1^{b}	29±1^{b}	31±1^{b}	F=13.169 p<0.001	3.1	-----
Duration of iu. treatment (days)[1]	55±5^{a}	35±3^{b}	33±3^{b}	32±4^{b}	F=8.730 p<0.001	10.6	-----
Number of iu. treatments[1]	9.6±0.7^{a}	4.7±0.6^{b}	3.8±0.4^{b}	4.1±0.5^{b}	F=22.417 p<0.001	1.64	-----
Number (%) of cows that conceived	14 (38%)^{a}	23 (61%)^{b}	16 (59%)	18 (62%)^{b}	-----	-----	$p_{(A-B)}=0.05$ $p_{(A-C)}=0.09$ $p_{(A-D)}=0.05$
Time of re-conception (day)[1,2]	113±9	94±6	90±7	89±8	F=1.986 p=0.12	----	-----

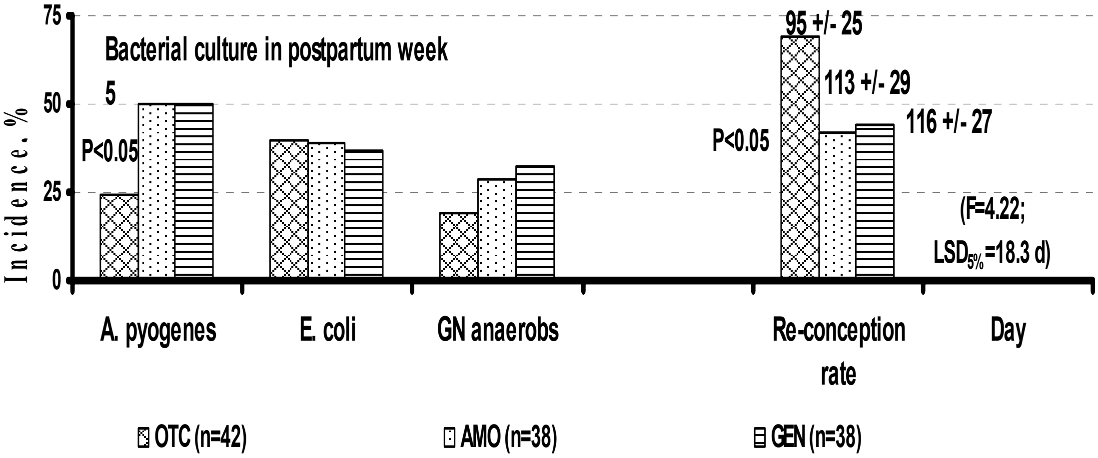
[1] Mean ± SEM
[2] In the case of cows that conceived within 150 days after calving
* the least significant difference on p=0.05 level
§ two-sample *t*-test p<0.001
a,b groups marked with different superscripts within the same row are significantly different from those marked with the same superscripts. Rows or groups without superscripts are not significantly different from one another.

Our results demonstrate that a correlation exists between decompensated negative energy balance manifesting itself in ketonuria and the course of involution complications. By antiketogenic treatment the efficacy of iu. antibiotic therapy can be improved and, consequently, the reproductive disturbances associated with puerperal metritis and the resulting economic losses can be reduced.

Trial 4

When the efficacy of treatments was studied on cows with PM (Trial 4a), the lochia (uterine discharge) lost its putrid character almost at the same time (on postpartum days 15±6, 14±4 and 13±4) and after an identical number of treatments (3.6±1.6, 3.3±1.2 and 3.2±1.2) in the three groups treated with oxytetracycline (OTC), amoxicillin (AMO) and gentamicin (GEN), respectively. The characteristic differences found between the treatment groups are shown in **Figure 2**.

Figure 2. Incidence rate of primary uterine pathogens in postpartum week 5 and re-conception rate of cows affected with puerperal metritis and treated with oxytetracycline, amoxicillin or gentamicin

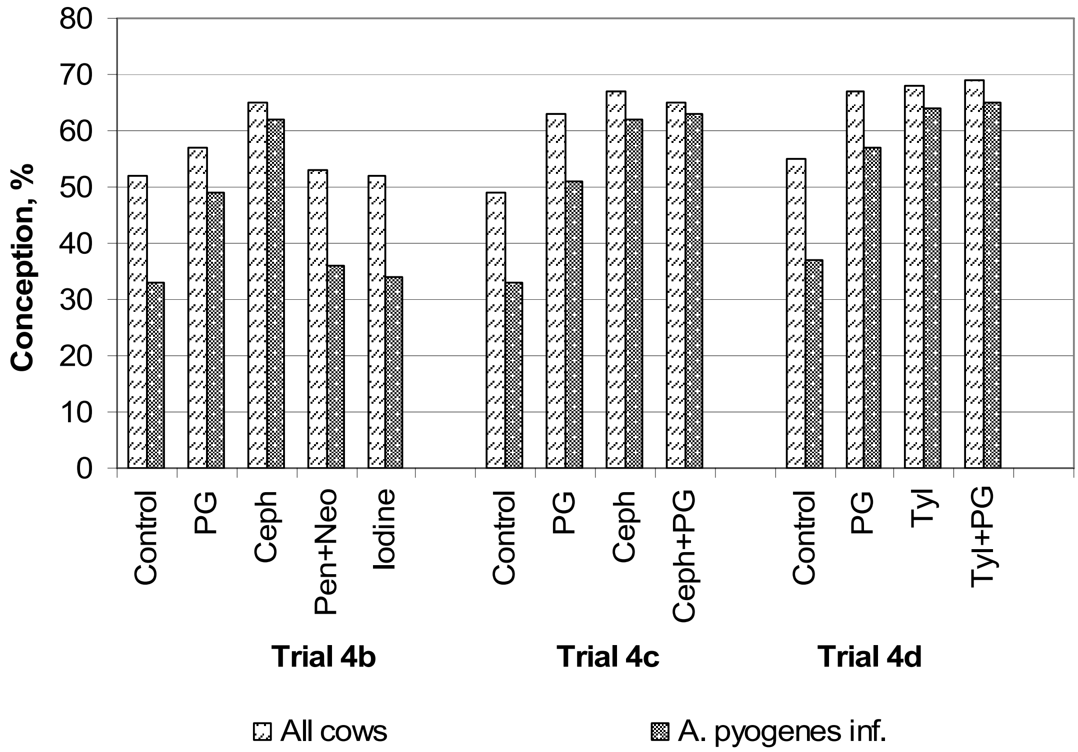


In the group treated with OTC, a smaller proportion of cows developed EM than in the groups treated with AMO and GEN (33%, 58% and 58%, separately; P<0.05). On postpartum days 28–35, *A. pyogenes* could be cultured from fewer cows and the re-conception rate was higher in the OTC-treated group than in the other two groups. However, none of the above treatments exerted an influence on the proportion of cows having uterine infection with *E. coli* or *Gram-negative anaerobes* even on postpartum days 28–35.

Efficacy of EM treatments (Trials 4b-4c-4d)

Studying the data of animals in the treatment groups *independently of the pretreatment bacteriological status,* in **Trial 4b** $PGF_{2\alpha}$ improved the conception rate by 5% while cephapirin increased it by 13%. In Trial 4c, the conception rate was improved by 14%, 18% and 16% by $PGF_{2\alpha}$, cephapirin and the combination of $PGF_{2\alpha}$ and cephapirin, respectively. A similar tendency was found in **Trial 4d** as well: the ratio of conceived cows increased by 12% after $PGF_{2\alpha}$ treatment, by 13% after im. tylosin treatment and by 14% after treatment with the tylosin + $PGF_{2\alpha}$ combination. However, differences between the groups were not statistically significant in any of the cases (**Figure 3**).

Figure 3. Re-conception rate of cows affected with clinical endometritis and treated with different medicinal products and of untreated control cows affected with clinical EM



Bacteriological examination of the uterus in postpartum week 5 demonstrated *A. pyogenes* infection in about half or two-thirds of the cows (52–69%). No major differences were found in the proportion of cows with uterine infection between the different experiments or treatment groups. Irrespective of the treatment applied, cows having uterine infection with *A. pyogenes* in postpartum week 5 (n=517) conceived in a lower proportion and later than their noninfected herdmates (n=354) (conception rate: 50% and 76%, respectively; $P<0.001$; time of re-conception: postpartum day 107±30 and 90±25, respectively; $P<0.001$). As compared to that of the untreated controls, as a result of cephapirin, tylosin and $PGF_{2\alpha}$ treatments the conception rate of cows with uterine infection caused by *A. pyogenes* significantly increased in all three experiments ($P<0.05$). The greatest increase was observed after treatment with cephapirin (29% in both experiments). Tylosin resulted in a 28% increase, while $PGF_{2\alpha}$ treatment produced a 16%, 18% and 20% increase, respectively, in the fertility rate of cows infected with *A. pyogenes*. Concomitant treatment with $PGF_{2\alpha}$ and either cephapirin or tylosin did not produce further improvement in the fertility rate. In contrast with the above findings, treatment with a penicillin-neomycin combination or with an inorganic iodine preparation had no influence on the conception rate as compared to the control **(Figure 3)**. None of the above treatments had notable effect on the length of the calving-to-conception interval.

On the basis of the treatment trials presented above, we can state that high-dose OTC is more effective for the treatment of PM than aminopenicillins (amoxicillin) or aminoglycosides (gentamicin). This higher efficacy manifests itself in a lower rate of *A. pyogenes* infection observed in postpartum week 5 and in a higher conception rate.

The presence of *A. pyogenes* in the uterus in postpartum weeks 4–5 adversely affects the fertility rate. Other bacteria isolated from the uterus do not have similar importance with regard to reproductive performance. Therefore, the detection of *A. pyogenes* in postpartum week 5 seems to be sufficient also in itself for monitoring the bacteriological status of the uterus. Effective antimicrobial therapy against this pathogen significantly improves the probability of conception in cows carrying *A. pyogenes* infection.

EM can be effectively treated with iu. cephapirin as well as im. tylosin or $PGF_{2\alpha}$. Although the above treatments were found to produce substantial improvement in the fertility rate of all cows irrespective of their bacteriological status, statistically significant difference was obtained only in the case of cows infected by *A. pyogenes*. At the same time, iu. penicillin-neomycin and inorganic iodine treatment did not prove to be sufficiently effective.

V. NEW AND NOVEL RESULTS OF THE DISSERTATION

1. We have constructed a model suitable for predicting the development of PM. Using this model in the practice, cows at risk of developing this disease can be identified in advance. Thus, the more frequent clinical examination of these cows would enable us to detect this disease as early as possible and to prevent or reduce its negative impact on reproduction.
2. We have demonstrated that the daily milk production decreases concurrently with the appearance of the pathognomonic sign of puerperal metritis (putrid uterine discharge), and that the rate of this milk production drop is greater in the more severe cases of PM.
3. The changes observed in recent years in the minimal inhibitory concentrations (MIC) of antimicrobials against aerobic bacteria (*E. coli, A. pyogenes*) causing metritis indicate reduced susceptibility of these bacteria to oxytetracyclines and amoxicillin. Although *E. coli* is not susceptible to cephapirin, this antimicrobial may be effective against *A. pyogenes*. Both pathogens showed adequate *in vitro* susceptibility to cefquinom.
4. We were the first to determine the minimal inhibitory concentrations of antimicrobials against anaerobic uterine pathogens isolated from uterine swab samples in Hungary (*Prevotella* group, *Bacteroides fragilis*, *Bacteroides gracilis* and *Fusobacterium* group). We have established that the MIC values of cephapirin and cefquinom were sufficiently low *in vitro*.

VI. USEFULNESS OF THE RESULTS FOR THE PRACTICE

1. As ketonuria is a good indicator of the negative energy balance of cows and can be detected by a simple method even under farm conditions, we recommend the use of this method on dairy farms in the postpartum period. In this way, antiketogenic treatment can be started in due time, which makes it possible to improve the negative energy balance. By this we can reduce the development of bacterial complications of uterine involution and, if diseases of involution have already developed, they can be cured more rapidly. As a result, the first insemination and fertilisation after calving can be performed earlier, which provides economic benefits for the dairy farms.
2. The daily milk yield decreases simultaneously with the appearance of the pathognomonic sign of puerperal metritis, i.e. an abnormal (putrid) uterine discharge. This reduced milk production calls attention to this disease, if the dairy farm has an equipment suitable for determining the milk quantity per milking. Knowledge of the time of appearance of putrid uterine discharge enables dairy operations to organise the examinations aimed at monitoring uterine involution in a manner that allows the diagnosis of all diseases.
3. On the basis of changes in the minimal inhibitory concentrations of antimicrobials against bacteria isolated from cases of metritis and relying upon our experience gained during the treatment trials we consider it necessary to re-evaluate the existing therapeutic protocols. For the treatment of puerperal metritis the traditionally applied tetracyclines have rather low efficacy nowadays, and recently the third- or fourth-generation cephalosporins have become the drugs of choice for this purpose. For the treatment of clinical endometritis, pyometra and subclinical endometritis, first-generation cephalosporins and/or $PGF_{2\alpha}$ and its analogues should be chosen.

In summary, we can state that since our studies have provided new and more precise information regarding the pathology and clinical features of the bacterial complications of postpartum uterine involution, their wider use on dairy farms would make it possible to improve the indices of reproductive performance, which is an essential condition of profitable operation.

VII. LIST OF PUBLICATIONS

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