FLUCTUATION OF NEUTROPHIL COUNTS AROUND PARTURITION IN HOLSTEIN DAIRY COWS WITH AND WITHOUT RETAINED PLACENTA

Pierangelo Moretti[a,b], Monica Probo[a,b], Andrea Cantoni[b], Saverio Paltrinieri[a,b,*], Alessia Giordano[a,b]

[a]Central Laboratory, Veterinary Teaching Hospital, University of Milan, via dell'Università 6, 26900 Lodi, Italy

[b]Department of Veterinary Medicine, University of Milan, via Celoria 10, 20133 Milan, Italy

*Corresponding Author at: Department of Veterinary Medicine – University of Milan, via Celoria 10, 20133 Milan, Italy. Tel.: ++39 02 50318103; Fax: ++39 02 50318095

e-mail address: saverio.paltrinieri@unimi.it (S. Paltrinieri)

Abstract

Retained placenta (RP) is often diagnosed in high-yielding dairy cows and can negatively affect reproductive performances. The objective of the present study was to investigate the hematological and biochemical profile of cows with RP before and immediately after parturition, with particular emphasis on neutrophil counts, since a previous study demonstrated the presence of peripheral neutropenia in dairy cows with RP sampled a few days after parturition. Results from 12 Holstein cows affected by RP and from 17 clinically healthy controls sampled one week pre-partum, within 12 hours after calving and between 48 and 72 hours after parturition were compared between groups and over time. Compared with controls, cows with RP had lower lymphocyte counts before parturition, lower leukocytes and neutrophils counts at parturition, lower monocytes counts at all times, and ~~slightly~~ higher β-hydroxybutyrate before and after parturition. Erythroid and biochemical parameters were similar ~~Similar variations~~ over time_ ~~were found~~ in both groups ~~for erythroid and biochemical parameters~~, whereas RP cows did not show the increase of neutrophil counts that occurs in controls at parturition. Hence, the finding of a lower neutrophil count in a routinely hemogram performed at parturition could be used as an alarm signal suggesting to monitor the affected animals. Moreover, although the underlying pathogenetic mechanism should be better investigated, the present study describes for the first time the association between altered blood leukocyte concentrations at parturition in RP compared to control cows.

Keywords: dairy cows, transition period, retained fetal membranes, peripheral neutrophils, production diseases

1. Introduction

The normal release of the placenta in cows is a multifactorial process due to a combination of hormonal, metabolic and immunological factors (Beagley et al., 2010). In particular, the activation of an innate immune response in the endometrium, mediated by the release of pro-inflammatory cytokines and chemokines, seems to contribute to the dissolution of the collagen link at the cotyledon-caruncle interface (Beagley et al., 2010; Davies et al., 2004). The alteration of anyone of the factors involved in the normal release may interfere with the whole process, leading to the occurrence of retained placenta (RP), a condition frequently observed in high-yielding dairy cows, that has been proven to cause negative effects on productive and reproductive performances (Dubuc et al., 2010; Kelton et al., 1998; Laven and Peters, 1996). In dairy cows, the negative energy balance (NEB) experienced during the transition period induces the activation of lipolytic metabolic pathways that are reflected by the increase of non-esterified fatty acids (NEFA) and ketone bodies in blood (Esposito et al., 2014). These molecules are responsible for direct and indirect effects on liver and immune cell functions that may determine on one side an inflammatory state and, on the other side, a suppression of immune responsiveness. Both these factors have been finally proven to increase the predisposition to production diseases as milk fever, endometritis, ketosis, displaced abomasum and retained placenta (Drackley, 1999; Sordillo and Mavangira, 2014). Several studies have shown that the decrease of neutrophil functions that characterizes the transition period is associated with or may predispose to the occurrence of RP (Gunnink, 1984a, 1984b; Kimura et al., 2002). Moreover, in a previous study we have found that cows with RP and without evidence of metabolic abnormalities and inflammatory conditions have lower circulating neutrophil counts soon after parturition compared with cows that do not experienced RP (Moretti et al., 2015a). However, in the cited study, hematological analyses were performed 3±1 days after parturition, when RP had just occurred, and it was thus not possible to clearly determine if the neutropenia was an early consequence of RP or a predisposing factor for its development. The objective of the present study

was to examine, through sequential blood samplings collected before and after parturition, the temporal dynamics of hematological and biochemical parameters around parturition in cows with and without RP, in order to better clarify the possible relationship between hematological and biochemical changes and the occurrence of this disease.

2. Material and methods

2.1 Study design, herds and groups

A prospective study was carried out on 4 intensive Holstein dairy farms located in the Po valley (Italy) from November 2013 to December 2014. The herds were composed of 270, 300, 700, and 300 animals, respectively, with 150, 130, 250 and 150 milking cows each. Herds were also characterized by mean days in milk (DIM) of 320, 180, 297 and 220 days, respectively, and a normalized production (average production adjusted for 305 days) of 9500, 10500, 11360 and 8900 kgs, respectively. All the cows were fed with a TMR (total mixed ration). Milking was performed twice a day, at 12 h intervals. A cow was judged to have an RP when the placental membranes had been retained for at least 24 h after parturition, in agreement with Fourichon et al. (2000), in order to have the certainty to exclude doubtful cases. All cows were monitored for the occurrence of vaginal discharge in the following 30 days. The electronic database of each farm was searched in order to retrieve information concerning health and management (clinical diseases, treatments, production, and days in milk) covering the study period.

Cows with RP and without other pathological conditions within the following 30 DIM were assigned to the RP group (n=12) whereas 17 cows, randomly selected within the cows with a normal parturition course, with fetal membranes released within 12 h (Peter, 2013) and without other pathological conditions in the following 30 DIM, were assigned to the control group (CTRL).

All the analyses performed in the present study were included in the routine laboratory panel for peri-partum monitoring so, according to the guidelines of our Institution, a formal approval from the Ethic Committee was not required.

2.2 Blood sampling

Peripheral blood samples from the coccygeal vein were collected in EDTA tubes (Venosafe plastic tubes for hematology, Terumo, Europe) and in plain tubes (Venosafe plastic tubes for serum, Terumo, Europe) ~~2 to 7~~7 to 2 days before parturition (T0), within 12 h after calving (T1) and between 48 and 72 hours after parturition (T2). ~~All the samples~~Both the samples in EDTA and those in plain tubes ~~-~~were immediately placed at 4 °C and submitted to the Central Laboratory of the Veterinary Teaching Hospital of the University of Milan where routine hematology was immediately performed as described below. Samples in tubes without anticoagulant were allowed to clot at room temperature for 30 minutes and then centrifuged at 2,500 g for 10 minutes. Harvested sera were then frozen at -80°C for a maximum of 3 months before biochemical tests were performed.

2.3 Hematology

Routine hematology was performed upon arrival at the laboratory after careful mixing of blood into the tubes, using an automated laser hematology analyzer (ADVIA 120 with multispecies software for veterinary use, Siemens Healthcare Diagnostics, Milan, Italy). The following variables generated by the instrument were recorded: hemoglobin (Hb) concentration, hematocrit (~~HCT~~Ht), erythrocyte (RBC) counts, total white blood cell (WBC) counts, mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), mean cellular volume (MCV), and

platelet (PLT) counts. The leukocyte differential provided by the instrument was checked microscopically on blood smears stained with a modified Romanowsky rapid stain (Dif-stain kit, Titolchimica S.P.A., Rovigo, Italy). The number of each leukocyte population was then calculated based on the total number of WBC and on the percentage of each cell population.

2.4 Clinical chemistry

In order to obtain information on biochemical analytes that may be associated with RP, routine biochemical analyses were run on serum with an automated spectrophotometer (ILAB300 plus, Instrumentation Laboratory S.p.a., Milan, Italy) using reagents provided by the manufacturer of the instrument, except when otherwise specified. The following analytes were measured: β-hydroxybutyrate (BOHB, D-3-Hydroxybutyrate dehydrogenase method, Randox Laboratories Ltd., Crumlin, Co. Antrium, UK), calcium (orthocresoftaleine method), creatinine (Jaffè method), glucose (GOD-POD method), non-esterified fatty acid (NEFA, ACS-ACOD method, Randox Laboratories Ltd., Crumlin, Co. Antrium, UK), phosphate (phosphomolibdate method), and total proteins (biuret method).

2.5 Statistical analysis

Within each sampling time (T0, T1 and T2) results from cows affected by RP and from CTRL group were compared using a non-parametric t-test for independent samples (Mann-Whitney U test~~).~~) since data did not have a normal distribution, based on a Kolmogorov-Smirnov test. Within the two groups (RP and CTRL) results were compared over time with a non-parametric ANOVA for paired samples (Friedman test) followed by a Wilcoxon signed rank test, when a significant difference was found. Statistical analyses were done on an Excel (Microsoft Corp, Redmond, WA,

USA) spreadsheet using the Analyse-it software (Analyse-it Software Ltd, Leeds, UK) with P value set at 0.05 for all calculations.

3. Results

3.1 Characteristics of the study population

A total of 111 cows were initially sampled during the study period (13, 46, 36 and 16 animals from herd A, B, C and D respectively). The incidence of RP among the sampled animals was 0% (0/13), 13% (6/46), 5.5% (2/36), and 25% (4/16) in herd A, B, C, and D respectively. No twins occurred. All the animals with RP were included in this study. Conversely, among the 99 cows that had normal parturition over the study period, only the 17 animals (3 form herd A and 14 from herd C) on which it was possible to collect and to properly process the complete sequence of samples (T0, T1 and T2) were included in the study. No significant differences between groups were found in terms of age (median age in the CTRL group = 3 years, min-max range = 2-7 years; median age in the RP group = 4 years; min-max range = 2-8 years) or in terms of numbers of lactation (median number in the CTRL group = 3, min-max range 1-7; median number in the RP group = 3, min-max range 1-5)

3.2 Hematology

Results concerning hematological parameters that had at least one significant difference among sampling times or between groups are graphically summarized in figure 1.

Concerning erythroid parameters, the analysis of results revealed that RP cows had significantly lower ~~HCT~~ Ht at T0 and significantly higher MCHC at all the time points compared to CTRL cows.

Concerning the comparison over time, RBC, Hb and ~~HCT~~ Ht significantly varied over time both in RP cows and CTRL cows, and the MCV only in controls. In all these cases values were significantly higher at T1 compared with the other two sampling times in both groups and slightly but significantly lower at T2 compared with T0 only in CTRL cows. Conversely, in CTRL cows the MCHC at T0 was significantly higher than others time points.

Concerning leukocytes, compared to CTRL cows, RP cows had significantly higher lymphocyte counts at T0 and significantly lower counts of total WBC and neutrophils counts at T1, eosinophils counts at T2 and monocyte counts at all the sampling times. Differences over time were found for total WBC and neutrophil counts only in CTRL cows, with a significant increase at T1 compared with the other sampling times. Both RP and CTRL cows showed significant decrease of eosinophils counts at T1 and T2 compared with T0.

3.3 Clinical chemistry

Results concerning hematological parameters that had at least one significant difference among sampling times or between groups are graphically summarized in figure 2.

Compared with CTRL cows, RP cows had significantly higher BOHB concentration at T0 and T2. In both RP and CTRL cows, compared with T0 calcium significantly decreased and NEFA significantly increased at T1 and T2, BOHB increased at T1, and creatinine decreased at T2. Glucose was significantly higher at T1 than in the other two sampling times in RP cows, while in CTRL cows a significant decrease at T2 compared with the other two groups was found. In CTRL cows the concentration of phosphate decreased at T1 and T2 compared with T0.

4 Discussion

The present study was designed to better understand the phenomenon of the peripheral neutropenia recorded in dairy cows with RP few days after parturition (Moretti et al., 2015a). To this aim, and especially to assess the temporal relationship between RP and neutropenia, sequential samplings were performed either before or immediately after parturition. The most important findings of the present study are the differences in leukocyte dynamics observed in CTRL cows compared with cows with RP. The increase of leukocyte and neutrophil counts and the decrease of eosinophil counts observed in cows that normally expelled the placenta may be justified by a normal response to the inflammatory and stressful stimuli necessary for the correct parturition course (Beagley et al., 2010). Conversely, lymphopenia, that is usually associated with a stress response in cattle (Tornquist and Rigas, 2010), was not observed. Differently to what happens in CTRL cows, leukocyte counts did not change across time in RP cows, that had also a lower number of circulating neutrophils and monocytes at parturition compared with cows without RP. In the previous study (Moretti et al., 2015a), neutrophil counts were lower in cows with RP compared with controls also in the first days after parturition, corresponding to sampling T2 of the current study, but this difference was not significant in the current study. However, a trend similar to that reported in the previous study was noticed also in the current study: median values were lower in RP cows than in controls, suggesting that the lack of significance may be a statistical artifact depending on the relatively low number of animals, coupled with the moderately high individual variability. The study design does not allow us to draw conclusions about the mechanism responsible for the lower number of neutrophils in the RP group or about the possible role of the lacking increased neutrophil count at T1 in the pathogenesis of RP. However, since in the previous study (Moretti et al., 2015a) an inflammatory response was excluded based on lack of increases in the serum level of inflammatory biomarkers, it may be postulated that the reduced availability of circulating neutrophils and monocytes at the time of parturition likely lead to an insufficient migration of these cells towards the endometrium in a phase when phagocytic cells are necessary for the proper dissolution of the collagen fetal-maternal link (Beagley et al., 2010; Davies et al., 2004, 2000).

Other minor findings concerning hematological parameters were also observed. Both cows with RP and controls showed an increase of the erythroid mass (increased RBC, Hb, and Ht) at parturition, probably as a consequence of stress and of a moderate dehydration associated with parturition (Bell, 1995). However, in both the groups, at the three sampling times, all the erythroid parameters values were within the reference intervals adopted in our laboratory for dairy cows at 3±1 DIM (Moretti et al.2015b), with only very few exceptions.

In order to assess whether neutropenia may be associated with biochemical changes, the serum concentration of analytes potentially involved in RP or in the reduction of neutrophil number of function were investigated. This analysis revealed very few significant differences in RP cows compared with controls: around parturition, high producing cows are subject to a negative energy balance (NEB) that favour lipomobilization and accumulation of NEFA in the blood (Opsomer, 2015). The results of this study showed that in both groups the rate of NEB was moderate, since the concentrations of NEFA and BOHB were within the reference intervals adopted in our laboratory for healthy cows at 3±1 days (Moretti et al., 2015b). However, although no differences over time or between groups were found for NEFA, cows that successfully expelled fetal membranes were able to maintain BOHB concentrations at a lower level compared with cows that retained the placenta. Several studies have proposed the role of hyperketonaemia as a possible cause of leukocyte dysfunction in ruminants: however very high blood concentrations of ketone bodies are required to induce this effect (Sartorelli et al., 2000, 1999; Scalia et al., 2006). Conversely, BOHB and acetoacetic acid at concentrations usually observed after parturition in dairy cows were shown to inhibit the proliferation of hematopoietic cells in the bone marrow (Hoeben et al., 2000). Although not investigated here, based on the values of BOHB recorded in the present study, it was unlikely that leukocyte functions were affected in RP cows, whereas the higher concentrations of BOHB observed in cows with RP before parturition may have influenced granulopoiesis in this group. This hypothesis need to be investigated through further studies aimed to assess the dynamic changes of leukocyte numbers in relation to ketone bodies in the transition period.

242 The role of calcium in the proper release of the placenta is still debated (Beagley et al., 2010). In the
243 study from Melendez et al. (2004), lower calcium concentrations were found six hours after
244 parturition in cows that retained the placenta, but in the present study no differences in calcium
245 concentration of RP and CTRL cows were found. A decrease in serum calcium was recorded in
246 both groups after parturition, likely depending on the increased secretion of calcium in colostrum
247 and on the inadequate ability of the cow to mobilize bone calcium to restore blood concentration in
248 the days after parturition (Martinez et al., 2012).
249 The few additional differences between RP and in CTRL cows for erythrocyte indices, glucose, and
250 phosphate levels were probably depending on statistical artefacts due to the individual variability,
251 since the trend of changes over time was similar in both groups.
252
253 **5. Conclusions**
254
255 In conclusion, most of the differences recorded over time both in RP and in CTRL cows likely
256 reflect similar metabolic and hormonal changes typical of the transition period. However, cows with
257 RP presented some important peculiarities around parturition, the most important of which was the
258 lack of the increase in neutrophil counts that occurs in control cows at parturition. In turn, the lower
259 neutrophil count may depend on a slightly higher concentration of BOHB. These changes were
260 detected before the occurrence of RP, possibly suggesting that they may potentially play a
261 pathogenic role in developing RP. Alterations of leukocyte functions associated with RP have been
262 widely discussed by different authors (Kimura et al., 2002; Gunnink, 1984a, 1984b). Conversely, to
263 our knowledge this is the first description of altered dynamics in blood leukocyte concentrations at
264 parturition in association with the occurrence of RP. However, additional studies should be
265 designed in the future to investigate the pathogenic mechanism of these changes and their possible
266 causative effects on RP. Regardless of the possible role of the lower neutrophil counts on the
267 pathogenesis of RP, this finding may have relevant diagnostic and prognostic implications in

268 routine practice, since a complete blood cell count, that is easy to perform and not expensive, may
269 provide helpful information. Even if attention was paid in order to select animals from herds with
270 similar management and production indices (all were intensive, high producing, commercial herds),
271 results of the present study should be interpreted taking into account the limited number of animals
272 included (in turn depending on the unpredictability of RP, that is a limiting factor in a prospective
273 study) and the possible influence of some unavoidable herd effect. Despite the above mentioned
274 limitations, these results suggest that, when hemograms are performed at parturition, the finding of
275 a low neutrophil count could be used as an alarm signal suggesting to closely monitor those cows as
276 possibly at risk to develop RP. Due to the lack of effective treatments for RP (Beagley et al., 2010),
277 this finding suggests that it would be worthy to sustain the aspecific immune system of cows in key
278 moments such as the pre-partum period, especially in herds with high prevalence of RP, as already
279 attempted by other authors (Kimura et al., 2014). Hence, future studies on a larger caseload should
280 be addressed to define the possible diagnostic threshold of WBC and neutrophil numbers that may
281 be used to predict the occurrence of RP, and to develop adequate strategies to further reduce
282 consequences on reproductive performances associated with prolonged retention of fetal
283 membranes.
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285 **Conflict of interest statement**

286 The Authors do not have any conflict of interest potentially influencing the results of this study
287

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Figures captions:

Figure 1: hematological parameters that were significantly different over time (T0 = 2 to 7 days before parturition; T1= within 12 h after calving; T2= between 48 and 72 hours after parturition) or between groups (CTRL = controls; RP = cows with retained placenta). Results of CTRL groups are indicated by white boxes, while results from RP cows are indicated by the grey boxes. The boxes indicate the I-III interquartile interval, the horizontal line corresponds to the median, vertical lines are the limits of outlier distribution according to the Tukey rule. Near outliers are indicated by the symbols "x" and far outliers with asterisks outside the boxes. The shaded grey area indicates the reference interval adopted in our laboratory for dairy cows at 3±1 days in milk. Bolded symbols within boxes indicated significant differences as follows: significant differences compared with T0 within the same group are expressed as * ($P<0.05$), ** ($P<0.01$), *** ($P\leq0.001$); significant differences compared with T1 within the same group are expressed as † ($P<0.05$), †† ($P<0.01$), ††† ($P\leq0.001$); significant differences in the RP group compared with the same time sampling of CTRL cows are expressed as ‡ ($P<0.05$), ‡‡ ($P<0.01$), ‡‡‡ ($P\leq0.001$).

Figure 2: biochemical parameters that were significantly different over time (T0 = 2 to 7 days before parturition; T1= within 12 h after calving; T2= between 48 and 72 hours after parturition) or between groups (CTRL = controls; RP = cows with retained placenta). Refer to figure 1 for the interpretation of boxes and symbols.