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The influence of L-carnitine on hematology and functional blood parameters of dairy cows with special focus on high resolution data around parturition

DISSERTATION
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LIST OF ABBREVIATIONS

(Accounts for the following Sections:

1. Introduction, 2. Background, 3. Scope of the thesis, 5. Discussion and 6. Conclusion)

°C	Degree Celsius
µl	Microliter
µm	Micrometer
ACA	Acetylcarnitine
ap	*Ante partum*
BCS	Body condition score
BHB	β-hydroxybutyrate
CAR	L-carnitine treated cows
CD	Cluster of differentiation
CoA	Coenzyme A
CON	Control group
CPTI	Carnitine palmitoyl transferase-I
DHR 123	dihydrorhodamine 123
DIM	Days in milk
DM	Dry matter
DMI	Dry matter intake
dROM	Derivatives of reactive oxygen metabolites
EDTA	Ethylenediaminetetraacetic acid
FACS	Fluorescence-activated cell sorting
FRAP	Ferric reducing ability of plasma
g	Gram

GPx	Glutathione-peroxidase
h	Hour
IL	Interleukin
l	Liter
LC	L-carnitine
M	Mol
MFI	Mean fluorescent intensity
min	Minute
ml	Milliliter
NEB	Negative energy balance
NEFA	Non-esterified fatty acids
PBMC	Peripheral blood mononuclear cells
PMN	Polymorphonuclear leukocytes
pp	*Post-partum*
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TG	Triglycerides
TLR	Toll-like receptor
TML	N-trimethyllysine
TNFα	Tumor necrose factor α
VLDL	Very low-density lipoproteins
γBB	γ-butyrobetaine

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With the exception of figures presented in Section 4.

1 GENERAL INTRODUCTION

Since 1990, the milk yield has increased from 4,692 to 8500 kg per year and cow (Brade and Brade, 2022). Although the life span of dairy cows has increased in recent years, a mean of only 36.7 months or 2.8 lactations is still common, and the maximum milk yield is naturally reached only during the fourth or fifth lactation period. Owing to the increasing metabolic challenges of the cows, there is an increased risk of performance-associated diseases such as mastitis, metritis, as well as fertility disorders and metabolic diseases, causing animals to leave significantly before the maximum milk yield. The latter may be associated not only with energy metabolism disorders, mineral balance, and fatty liver syndrome, but also infectious-inflammatory processes, and a suppressed immune system or oxidative stress. Therefore, the short lifespan of dairy cows is critical for economic and ethical reasons and is not sustainable. To improve the situation of dairy cows on farms worldwide, numerous feeding trials have been conducted to enhance cows' ability to adapt to the transition period and, therefore, to support animal health.

As observed in previous studies, there are individual differences in the ability of cows to cope better with the critical phase of the transition period. This critical transition period can be improved by optimizing the immunological setting and reducing oxidative stress. To improve this situation, good energy metabolism is necessary to ensure that immune cells are sufficiently supplied with energy. Therefore, the functionality of mitochondria, the "power house" of cells, plays a major role and here particularly β-oxidation by transporting fatty acids from the cytosol into the mitochondria matrix via carnitine shuttles (Schlegel et al., 2012; Ringseis et al., 2018). Furthermore, mitochondrial dysfunction can lead to a pro-inflammatory condition, which consequently stresses the cow's immune system during the transit phase (Viscomi et al., 2015). In summary, well-functioning mitochondria can, on the one hand, improve the energy situation of cells, and thus of cows, and on the other hand, also support immune function (Walker et al., 2014).

In this respect, the present PhD thesis evaluated the influence of an L-carnitine (LC) supplementation throughout the transition period of dairy cows on the blood cell count, functionality of blood leukocytes, and antioxidant parameters in the blood serum. A special focus will be on the timespan of the first 72 h directly after calving, which, to the best of our knowledge, has been observed for the first time at such a high resolution.

2 BACKGROUND

2.1 Joint research project MitoCow

This work was a part of the joint research project MitoCow, supported by the German Research Foundation (DFG). One important objective of this project was to evaluate the factors influencing mitochondrial functionality in dairy cows. All cows were investigated for calving as an individually variable stimulus on the one hand and a standardized stimulus of an inflammatory metabolic challenge on the other. Half of the dairy cows were supplemented with LC to investigate its potential effects on performance, health, and mitochondrial functionality. The information obtained should help select healthier and better-performing cows for milk production and reduce welfare-related health problems.

The experiment was conducted at the Friedrich-Loeffler-Institute, Brunswick, Germany. The physiological, pathophysiological, and immunological effects of feeding and challenge were characterized with respect to dietary LC supplementation.

Part of this thesis aimed to investigate and further characterize the physiological and pathophysiological effects on hematology, immune function, and antioxidant capacity, as well as the influence of nutrition, with a special focus on the first 72 h after calving.

The participating partners were the Department of Functional Anatomy of Livestock and the Department of Feed-Gut Microbiota Interaction of the Institute of Animal Science of the University of Hohenheim, Germany; the Institute of Animal Sciences of the University of Bonn, Germany; and the Clinic for Cattle of the University of Veterinary Medicine, Hannover, Germany. Other research facilities have investigated the microbiome, metabolome, and gene expression in the liver.

2.2 Challenges around parturition

The transition period, defined as three weeks before (*ante partum*, *ap*) until three weeks after parturition (*post-partum*, *pp*), is a challenging and critical time period in the lifespan of cows, caused by the change from a pregnant non-lactating state to a non-pregnant lactating condition (Drackley et al., 2001; LeBlanc, 2020). The most critical are parturition itself, reduced feed intake around calving, and the onset of lactation, which suddenly increases energy and mineral requirements. The consequences can be categorized into five critical phenomena, which, however, influence each other:

- Negative energy balance (NEB)
- Hypocalcemia
- Reduced immune competence
- Systemic inflammatory reaction without microbial or pathological etiology
- Oxidative stress

Most changes occur directly after parturition except for the alteration of immune competence, which is sometimes observed before calving (Kehrli, M. E., Jr. et al., 1989; Goff and Horst, 1997; Lacetera et al., 2005). Understanding the origin of these changes and the timeframes of their occurrence in relation to calving appears to be critical for discovering the turning point in homeostasis during the transition period. By defining and characterizing the physiological processes around calving, it is possible to gain deeper insight into and positively influence these phenomena, thus helping cows better through the transit period.

The following section describes NEB, reduced immune competence, systemic inflammation, and oxidative stress in more detail. Hypocalcemia is not described below as it is not relevant to this thesis.

2.2.1 Negative energy balance

During the transition period, cows must cope with adjustments such as fetal growth, galactopoiesis, lactogenesis, hormonal changes, and increasing energy and nutrition demands. Galactopoiesis is the maintenance of milk production, and lactogenesis is the formation of the mammary gland. The latter is one of the most critical issues for periparturient dairy cows. Before calving, dry matter intake (DMI), and consequently, blood glucose levels decrease (Grummer, 1993). Furthermore, pancreatic insulin production decreases pp and further induces glucose utilization in insulin-sensitive organs (Drackley et al., 2001). Due to reduced energy intake, cows enter a temporary state of NEB and start to mobilize body mass, primarily adipose tissue, to cope with the energy deficit (Bell, 1995). In parallel with increased lipolysis, non-esterified fatty acid (NEFA) concentrations in the blood rise, and NEFA reaches the liver by circulating throughout the body. In the liver, NEFA is completely oxidized via β-oxidation to acetyl-CoA. If insufficient oxalate is present, NEFA is converted into triacylglycerides (TG). TG is exported via very low-density lipoproteins (VLDL) into the blood and stored in the liver or mammary glands for milk fat synthesis. In early lactation, the rapid and massive flux of NEFA to the liver and the limited amount of oxaloacetate, which is needed as a substrate for gluconeogenesis and thus lactose production, lead to an increase in the synthesis of ketone bodies like β-hydroxybutyrate (BHB) from acetyl-CoA. In addition, the increasing concentrations of acetyl-CoA inhibit the breakdown of NEFA, which is then re-esterified

to TG. Increased NEFA, BHB, and TG levels can lead to metabolic diseases, such as ketosis or fatty liver syndrome (Grummer, 1993).

Increased tissue mobilization has also been shown to have adverse effects on the immune system (Cai et al., 1994), owing to increased concentrations of NEFA and BHB, which negatively affect the function of immune cells around birth and early lactation (Wentink et al., 1997; Suriyasathaporn et al., 1999; Hammon et al., 2006). Therefore, high blood NEFA and BHB levels are risk factors for mastitis and uterine diseases (Hammon et al., 2006; Moyes et al., 2009). It can be concluded that lower concentrations of NEFA and BHB around calving could improve the energy situation and thus might ameliorate the cows' health.

2.2.2 Reduced immune competence – Immunosuppression

Parturient cows must deal with a state of immune suppression induced by many different factors, such as NEB, fat mobilization (Lacetera et al., 2005), production of reactive oxygen species (ROS) (Sordillo and Aitken, 2009a), ketosis (Bertoni et al., 2009), and various hormones that are important during pregnancy and parturition, such as progesterone, estrogen, and glucocorticoids (Meglia et al., 2005; Vlasova and Saif, 2021).

Birth is initiated through the release of adrenocorticotropic hormones (ACTH) and glucocorticoids. This initiate hormonal changes in the dams. Progesterone levels should be high to maintain pregnancy. With increased levels of ACTH and glucocorticoids emanating from the calf, progesterone is converted into estrogen (Senger, 2004). This causes the progesterone and estrogen levels to decrease and increase, respectively (Fig. 1). At the same time, the cow's cortisol levels also increase (Hudson et al., 1976; Hydbring et al., 1999). These hormonal changes cause changes in cellular immune and inflammatory responses (Fig. 1).

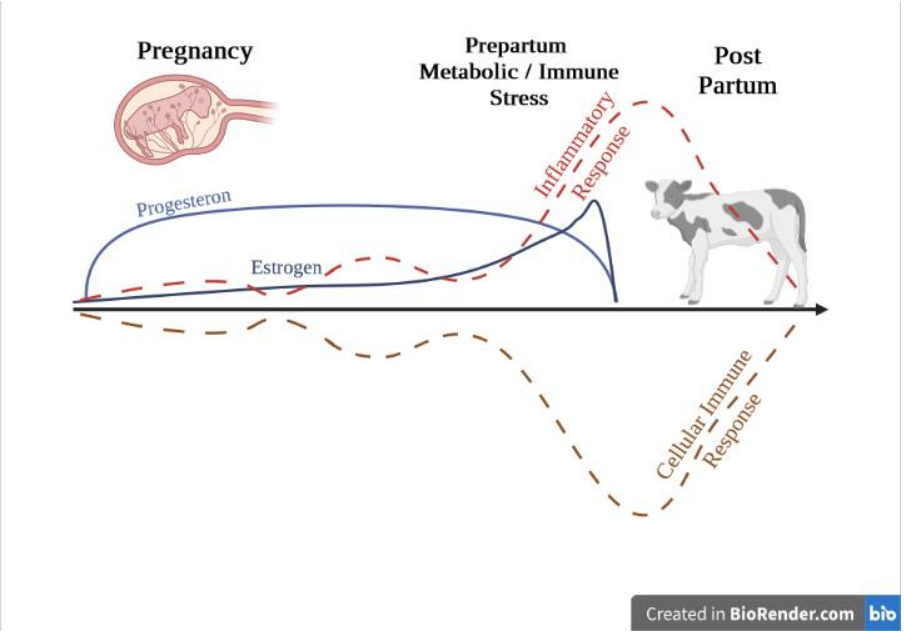

Figure 1: Immune modulation during pregnancy of dairy cows. Adapted from Vlasova and Saif (2021). Created with BioRender.

The reduced cellular immune response manifests as a decrease in eosinophil granulocytes and lymphocytes, such as T-helper cells ($CD4^+$) and cytotoxic T-cells ($CD8^+$), in the blood (Menge and Dean-Nystrom, 2008; Oliveira et al., 2012).

In addition to alterations in the number of immune cells around calving, other hematologic parameters, such as those of platelets, also change. This, in turn, plays an important role because platelets, in addition to their function in hemostasis, also interact with immune cells. Human platelets contain Toll-like receptors (TLR) (Cox et al., 2011). Furthermore, some effects of eosinophil granulocytes and platelets have been described, including the production of signaling molecules for the coagulation system mediated by eosinophils (Hogan et al., 2008; Abdala-Valencia et al., 2018; Coden and Berdnikovs, 2020).

Basically, it should be mentioned that in this thesis, the term “reduced cellular immune response” corresponds to the term “immunosuppression” around calving. Changes in estrogen and progesterone levels lead to altered lymphocyte and granulocyte functions (Roth et al., 1982; Lamote et al., 2006). Additionally, progesterone alters immune cell populations and functions to promote immune tolerance by decreasing the expression of major histocompatibility (MHC) proteins in trophoblasts and altering tissue remodeling and angiogenesis (Ott, 2019). Consistently high levels of progesterone during pregnancy result in an immune balance shift from T-helper cells 1 (Th1) to T-helper cells 2 (Th2) and its maintenance until the periparturient period (Maeda et al., 2013; Paibomesai et al., 2018). However, at parturition, the Th1/Th2 ratio should change rapidly from tolerance for the fetus (high Th2) to protection against infectious agents (high Th1) (Trevisi and Minuti, 2018). There is also evidence that $CD4^+$ (T-helper cells) and $CD8^+$ (cytotoxic T-cells) cells

in the peripheral blood decrease, whereas $CD4^+CD25^+$ cells (regulatory T-cells) increase, suggesting immune suppression during the peripartum period in cows (Oliveira et al., 2012). The shift towards a Th2 immune response should also lead to an increase in B cells before calving. After calving, the number of B cells decreases again, owing to a new shift in the Th1/Th2 immune response (Vlasova and Saif, 2021).

Increased glucocorticoid levels around parturition alter the cellular functions of blood leukocytes as well as the number of circulating polymorphonuclear cells (PMN) (Burton et al., 1995). Glucocorticoids inhibit the activation and migration of granulocytes into tissues, leading to neutrophilia (Zerbe et al., 2000; Meglia et al., 2005) by downregulating L-selectin expression (Burton et al., 1995).

The immune system is also influenced by reduced DMI and the resulting decrease in glucose levels and the concurrent increase in NEFA and BHB. Because glucose is the preferred energy source for immune cells, lower glucose availability hampers the maintenance of cellular functions and impairs the triggering of optimal host responses. A decreased glucose uptake has been shown to reduce the phagocytic capability in mice (Barghouthi et al., 1995). NEFA and BHB can also be used as energy fuels by immune cells; however, it has been demonstrated that during inflammation, glucose is the preferred metabolic fuel for activated PMN, macrophages, and lymphocytes (Barghouthi et al., 1995; Pithon-Curi et al., 2004). NEFA and BHB inhibit different functions of the immune system. BHB decreases phagocytosis and oxidative bursts (Hoeben et al., 1997; Sartorelli et al., 1999). NEFA also impairs cytokine production, cell viability, phagocytosis, and antigen presentation (Lacetera et al., 2004; Scalia et al., 2006).

In addition to the effects of parturition on immune functions, oxidative stress development during parturition is also a factor that leads to the dysfunction of immune responses and inflammatory reactions. This is described in more detail in Section 2.2.3.

As a consequence of these influences on the cellular immune response, the risk of infection increases in dairy cows around calving. Depression of several immune functions reported around parturition manifests itself in a higher prevalence of clinical mastitis and other diseases during the transition (Oliver and Sordillo, 1988).

2.2.3 Oxidative stress

During the last trimester of gestation and parturition, increased metabolic activity leads to enhanced oxygen demand, in parallel with the accumulation of ROS (Sordillo and Aitken, 2009a). ROS are typical byproducts of the respiratory chain (Valko et al., 2007). During parturition, there is an imbalance between pro-oxidant and antioxidant forces (Gitto et al., 2002). Stress hormones such as

cortisol and inflammatory processes during parturition additionally trigger oxidative stress (Bionaz et al., 2007; Trevisi et al., 2012). ROS are effective signaling molecules involved in signal transduction and are essential for regular cellular processes (Franchina et al., 2018). Furthermore, ROS promotes inflammatory processes and initiates antioxidant defenses.

Important antioxidative enzymes for ensuring the balance between oxidative stress and anti-oxidative capacity are the superoxide-dismutase (SOD) and glutathione-peroxidase (GPx) (Konvičná et al., 2015). These enzymes include selenium, copper, zinc, and manganese in their catalytic sites. These trace elements can be reduced in the plasma around calving because of the decreased DMI, which results in decreased antioxidative defense (Meglia et al., 2001). Although cows consume antioxidants in their diets, decreased DMI and the parallel export of minerals, vitamins, and trace elements into the colostrum and milk lead to an unbalanced antioxidative system (Spears and Weiss, 2008; Sordillo and Mavangira, 2014). Oxidative stress occurs in combination with increased ROS production.

2.2.4 Systemic inflammation – inflammatory response

Around the time of parturition, many different factors play key roles in causing the body to respond to a stressful and energy-consuming challenge, which is comparable to responses to systemic inflammation without signs of microbial infection and/or otherwise determined pathology (Sordillo et al., 2009b; Bionaz et al., 2007; Bertoni et al., 2008). During parturition, the inflammatory response increases (Fig. 1). One sign of systemic inflammation is a peak in acute-phase proteins (APP) shortly after calving. APPs are part of the nonspecific immune defense system and are released into the blood in response to tissue damage or acute inflammation. The reasons for inflammatory events during and after calving are diverse and interconnected with the critical events mentioned previously.

During pregnancy, immunosuppression is desired to ensure that the fetus is tolerated (Oliveira et al., 2012). However, after birth, the fetal placenta must be rejected by inflammatory processes (Mordak et al., 2015). Uterine and vaginal tissues are injured at birth (Trevisi and Minuti, 2018), triggering inflammatory reactions. In addition, microorganisms and bacteria can easily penetrate injured locations and trigger inflammatory reactions in the mammary glands, uterus, and birth canal (Burvenich et al., 1999; LeBlanc, 2010).

Oxidative stress and increased production of ROS during parturition increase inflammatory response and expression of pro-inflammatory mediators by activating redox-sensitive transcription factors, which in turn increases oxidative stress (Sordillo et al., 2009b).

2.3 Altered oxygen demand during the transition period

During pregnancy, particularly in the last trimester, the dam's body undergoes physiological changes to provide optimal care for herself and the calf. Thus, for milk production after birth, mammogenesis, lactogenesis, and galactopoiesis take place (Akers, 2017). These processes are characterized by cell proliferation and tissue expansion. Furthermore, the oxygen demand of cows increases, particularly during the last trimester of pregnancy. On the one hand, the dam must provide herself and the unborn calf with oxygen; on the other hand, the mammary gland must be supplied with oxygen throughout lactogenesis and galactopoiesis, and the dam's demand for ATP increases. Increased blood flow and oxygen transport capacity This is regulated by adjustments in cardiac output and an increased respiration rate. During this adaptation process, local and systemic hypoxia initially occurred. This triggers the expression of hypoxia-inducible factor 1 (HIF), which is involved in regulating different cellular processes. Around calving, HIF triggers angiogenesis and erythropoiesis, providing a better oxygen supply by inducing the release of erythrocyte precursors from the bone marrow (Moritz et al., 1997; Fandrey, 2004; Shao and Zhao, 2014). Studies in cows and goats showed a positive correlation between mammary blood flow and mammary oxygen uptake, as well as milk yield (Fleet and Peaker, 1978; Götze et al., 2010). It had been found that the Late exponential fetal development and mammogenesis lead to an oxygen deficit and induce the expression of HIF and EPO (Moritz et al., 1997; Fandrey, 2004). Stimulated erythropoiesis is reflected in erythrocyte anisocytosis and a peripheral increase in erythrocyte size variation (RDW). Polycythemia and macrocytosis occur simultaneously with erythropoiesis. This was indicated by an increase in the capacity of HGB to bind oxygen, as seen in the elevated proportion of oxygenated HGB. Oxygenated HGB tends to increase the rate of HGB autoxidation (Abugo and Rifkind, 1994) and, in parallel, raise the affinity of partially oxygenated HGB for RBC membranes. This may lead to increased ROS formation within the membrane, resulting in peroxidative damage (Nagababu et al., 2008) and, consequently, compromised erythrocyte functionality. To prevent peroxidative damage to the cells, erythrocytes express enzymes that convert superoxide anions into hydrogen peroxide. In addition to antioxidant enzymes, non-enzymatic antioxidants are endogenously synthesized and exogenously absorbed. Thus, as described below, animals ingest antioxidants such as vitamins (B2, C, and E), minerals (selenium and zinc), and LC.

2.4 L-carnitine

Carnitine is an obligatory quaternary amine synthesized from lysine and methionine. In general, carnitine exists in two forms, D- and L-carnitine; however, only the L-isomer is physiologically active (Bieber, 1988; Uluisik and Keskin, 2014). L-carnitine (LC) can be found in plants, bacteria,

and animals. LC status in animals depends on endogenous synthesis, absorption via the gastrointestinal tract as well as excretion (Ringseis et al., 2018). LC is synthesized using N-trimethyllysine (TML). For this purpose, TML is converted in various enzymatic steps into γ-butyrobetaine (γBB), the direct precursor of LC. This is then converted into liquid crystals (LC) via hydroxylation. (Vaz and Wanders, 2002). Endogenous synthesis occurs in the kidneys, liver, and skeletal muscles (Vaz and Wanders, 2002). It contributes to the regular cellular functions of all mitochondria-containing cells. In combination with activated acyl residues, LC form acylcarnitines, which are mediated by carnitine palmitoyl transferase-I (CPT1), to shuttle fatty acid residues from the cytosol into the mitochondrial matrix (Schooneman et al., 2013), which is the site of fatty acid β-oxidation. Therefore, it can be assumed that a lack of LC leads to an insufficient energy supply and, consequently, mitochondrial dysfunction. This, in turn, could lead to a problem in case of an increased energy demand since not enough energy can be provided by the mitochondria. LC also plays an important role in regulating free Coenzyme A (CoA) by regulating the mitochondrial acyl-CoA/CoA ratio and buffering the free CoA pool (Ramsay and Arduini, 1993). Through the modulation of available free CoA, carnitine also influences energy production from glucose (Stephens et al., 2007). However, this condition might be circumvented by LC supplementation, leading to improved mitochondrial efficiency and the prevention of dysfunction.

Next to the mentioned functions, LC also has properties of a dietary antioxidant by protecting antioxidant enzymes from further peroxidative damage. Furthermore, LC and its derivatives protect cell membranes from ROS (Gülçin, 2006). The protective effects of LC on membrane stability and lipid oxidation have been detected in vitro and in vivo in different cell types, e.g., platelets and erythrocytes (Pignatelli et al., 2003; Sweeney and Arduini, 2004; Saluk-Juszczak et al., 2010; Uluisik and Keskin, 2014).

Considering the aforementioned effects of LC on energy metabolism and antioxidative functions, it is a highly promising supplementation candidate in the periparturient period, where all the abovementioned factors influence and lead to dysfunctional immune responses in transitional dairy cows. LC ameliorates immunosuppression in these animals.

3 SCOPE OF THE THESIS

The literature has suggested that immune system dysfunction prior to and immediately after calving is due to a combination of endocrine and metabolic factors. However, it is unclear whether reduced immunocompetence is a physiological condition in dairy cows and/or an early sign of disease triggered by other events, such as NEB or pathogen entry. In either case, the effects of this impairment became most apparent after calving. To elucidate the reason for this immune impairment, it is necessary to understand the onset of changes in immune function during gestation. Furthermore, LC, which has the potential to enhance the energy status and exert antioxidative and membrane-stabilizing effects, could support energy metabolism and immune responses during the transition period of dairy cows. The efficiency of mitochondrial function may be a critical factor affecting all cells that modulate individual animal adaptability during the transition from pregnancy to lactation.

Therefore, the present study aims to characterize the hematological and immunological profiles around calving more precisely and determine if LC supplementation under these difficult physiological conditions positively affects the energy metabolism of cows and their cells and, thus, the immunological status. Particular attention will be on the period immediately after calving. Therefore, cows were sampled at very short intervals, especially within the first 72 h pp. The animal trial was conducted from 42 days ap until 110 days pp according to Meyer et al. (2020). Rations contained increasing concentrate proportions of 30%–50% on a dry matter (DM) basis and roughage (70% maize silage and 30% grass silage) within days of calving. Cows in the L-carnitine-treated group (CAR) received 25 g per cow per day of rumen-protected L-carnitine (CAR; n=30). The remaining animals (control group (CON), n=29) received an equivalent amount of fat (BergaFat F-100 HP; Berg-Schmidt GmbH & Co. KG; Hamburg, Germany).

The findings of these investigations are reported in the following three publications and are furthermore discussed comprehensively in Chapter 5, “General Discussion.”

Manuscript I addresses the influence of LC supplementation on energy status around calving, emphasizing the energy-consuming puerperium, including recovery from calving and the magnitude of NEB. For this purpose, the performance, metabolic state, energy efficiency, biochemical status, and clinical health of cows were determined.

Manuscript II focuses on the effects of LC on red blood cell count, platelet count, related parameters, and antioxidative capacity in serum/plasma (FRAP, D-ROM, SOD, and GPX enzyme activity).

Manuscript III outlines the effect of LC supplementation on the number and characterization of different leukocyte populations, as well as on the phagocytic capacity and ROS production of immune cells.

4 MANUSCRIPTS

The following section contains the manuscripts, which were included in the present thesis. All manuscripts were published in international peer-reviewed journals. The manuscripts are presented within this thesis in the latest version of the text during the respective submission process. Text layout and formatting was adapted for the present thesis.

I Effects of a Dietary L-Carnitine Supplementation on Performance, Energy Metabolism and Recovery from Calving in Dairy Cows.

Published in *Animals* **10** (2), 342 (2020)

II Effects of Dietary L-Carnitine Supplementation on Erythrogram and Platelets of Dairy Cows with Special Emphasis on Parturition.

Published in *Dairy* **2**(1), 1-13 (2021)

III Dietary L-Carnitine Affects Leukocyte Count and Function in Dairy Cows Around Parturition.

Published in Frontiers in Immunology **13**, 1-17 (2022)

I

Effects of a Dietary L-Carnitine Supplementation on Performance, Energy Metabolism and Recovery from Calving in Dairy Cows

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Simple Summary

Dairy cows develop metabolic diseases especially in the transition period due to high energy requirements for the process of calving, beginning milk production and, simultaneously, restricted feed intake capacity. L-carnitine is endogenously synthesised as an obligatory, quaternary amine for the initial step of ß-oxidation, but with the onset of lactation it is also excreted with milk, whereby its availability for other metabolic pathways might be limited. Supplemental L-carnitine might be able to fill in this apparent gap and to enhance the efficiency of ß-oxidation, whereby the magnitude of negative energy balance would be decreased. The present experiment mainly focused on the energy-consuming process of calving itself and on the energy metabolism during the first weeks of lactation.

Abstract

Dairy cows are metabolically challenged during the transition period. Furthermore, the process of parturition represents an energy-consuming process. The degree of negative energy balance and recovery from calving also depends on the efficiency of mitochondrial energy generation. At this point, L-carnitine plays an important role for the transfer of fatty acids to the site of their mitochondrial utilisation. A control (n = 30) and an L-carnitine group (n = 29, 25 g rumen-protected L-carnitine per cow and day) were created and blood samples were taken from day 42 ante partum (ap) until day 110 post-partum (pp) to clarify the impact of L-carnitine supplementation on dairy cows, especially during the transition period and early puerperium. Blood and clinical parameters were recorded in high resolution from 0.5 h to 72 h pp. L-carnitine-supplemented cows had higher amounts of milk fat in early lactation and higher triacylglyceride concentrations in plasma ap, indicating increased efficiency

Keywords: L-carnitine; dairy cow; performance; energy metabolism; lipomobilisation; clinical score; calving; parturition

II

Effects of Dietary L-Carnitine Supplementation on Erythrogram and Platelets of Dairy Cows with Special Emphasis on Parturition.

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Abstract

During late gestation and early lactation, many proliferative processes and metabolic adaptions are involved in homeorhesis. An adjusted supply of oxygen is a pre-condition for an optimized cellular energy metabolism whereby erythrocytes play a central role. Endogenous L-carnitine modulates the mitochondrial fatty acid utilization for generating adenosine triphosphate (ATP). As it might be insufficient around calving due to increased need, L-carnitine supplementation is frequently recommended. Thus, the present study addressed the interplay between the red hemogram, platelets, oxidative stress indices, and L-carnitine supplementation of dairy cows around calving. German Holstein cows were assigned to a control (n = 30) and an L-carnitine group (n = 29, 25 g of rumen-protected L-carnitine per cow and per day), and blood samples were taken from day 42 ante partum (ap) until day 110 postpartum (pp), with a higher sampling frequency during the first three days pp. The time courses of the erythrogram parameters reflected the physiological adaptations to the oxygen need without being influenced by L-carnitine supplementation. Erythrocytic antioxidative enzymatic defence paralleled the relative development of polycythemia ap, while non-enzymatic total plasma antioxidative capacity continuously increased pp. In contrast to erythrocytes, the platelet counts of the L-carnitine supplemented cows varied at significantly higher levels. This can be interpreted as a result of a membrane-stabilizing effect of L-carnitine.

Keywords: L-carnitine; dairy cow; hematology; erythrocytes; erythrogram; platelets; calving; parturition

III

Dietary L-Carnitine affects leukocyte count and function in dairy cows around parturition.

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Abstract

In early lactation, an energy deficit leading to a negative energy balance (NEB) is associated with increased susceptibility to disease and has been shown to be an important factor during transition in dairy cows. L-carnitine as a key factor in the mitochondrial transport of fatty acids and subsequently for β-oxidation and energy release is known to modulate mitochondrial biogenesis and thus influence metabolism and immune system. In the current study, we characterized hematological changes around parturition and investigated the potential effects of dietary L-carnitine supplementation on immune cell functions. For this approach, dairy cows were assigned either to a control (CON, n = 30) or an L-carnitine group (CAR, n = 29, 25 g rumen-protected L-carnitine per cow and day (d)). Blood samples were taken from d 42 ante partum (ap) until d 110 post-partum (pp), with special focus and frequent sampling from 0.5 to72 h post-calving to clarify the impact of L-carnitine supplementation on leukocyte count, formation of reactive oxygen species (ROS) in polymorphonuclear cells (PMN) and peripheral mononuclear cells (PBMC) and their phagocytosis activity. Blood cortisol concentration and the capacity of PBMC proliferation was also investigated. All populations of leukocytes were changed during the peripartal period, especially granulocytes showed a characteristic increase up to 4 h pp. L-carnitine supplementation resulted in increased levels of eosinophils which was particularly pronounced one day before to 4 h pp, indicating a possible enhanced support for tissue repair and recovery. Non-supplemented cows showed a higher phagocytic activity in PBMC as well as a higher phagocytic capacity of PMN during the most demanding period around parturition, which may related to a decrease in plasma levels of non-esterified fatty acids reported previously. L-carnitine, on the other hand, led to an increased efficiency to form ROS in stimulated PMN. Finally, a short period around calving proved to be a sensitive period in which L-carnitine administration was effective.

Keywords: L-carnitine; dairy cow; Parturition; phagocytosis; ROS production; leukocyte functionality

5 GENERAL DISCUSSION

The present study aimed to characterize the period around calving more precisely, especially with regard to hematology and immunology, and investigate the effects of LC supplementation (25 g/animal/day) from day 42 ap to day 110 pp. The focus was on the first 72 h after calving. The following parameters were included: performance, energy efficiency, animal health, clinical chemistry, hematology, antioxidant enzymes, and functional capacity of immune cells. During the transition period, dairy cows need to adapt from a pregnant non-lactating to a non-pregnant lactating status (Drackley et al., 2001; LeBlanc, 2020) characterized by dramatic physiological changes and adaptation processes in metabolism, hormones, and the immune system.

The main effects of LC supplementation and parturition are discussed below and are related to hematology, immune function, and the effect on oxygenation.

Supplementation with 25 g of rumen-protected LC per day and cow significantly increased the plasma concentrations of γBB, acetylcarnitine (ACA), and LC in CAR cows compared to those in CON cows (Manuscript I). TML and γBB are both precursors in the endogenous synthesis of LC; only γBB was affected and increased in the CAR group compared to the CON group over the entire trial period. This may be because TML was converted into γBB; however, in the CAR group, it was not converted into LC, as LC was already sufficiently available due to the supplementation. The time period until 14 days ap, when increased γBB was observed (Manuscript I), can probably be regarded as the time needed for adaptation to LC supplementation. The decrease in plasma concentrations of γBB and LC from day 14 ap until 0.5 h pp in both groups could indicate alterations in the LC metabolism during this period independent of LC supplementation. The hepatic mRNA abundance of genes involved in fatty acid uptake, fatty acid oxidation, ketogenesis, and enzymes involved in LC synthesis, as well as LC uptake, increased from week 3 ap to week 1 pp in dairy Holstein cows, indicating that liver cells received sufficient amounts of LC for the transport of excessive amounts of NEFA (Schlegel et al., 2012). It has been shown that LC is metabolized and released primarily as ACA from liver cells into the bloodstream (Christiansen and Bremer, 1976), which explains the persistent increase of ACA concentration, when concentrations of γBB and LC decreased in the blood but probably increased in liver cells (Schlegel et al., 2012). In the present trial, however, LC and metabolite levels were only analyzed in the blood, and the intracellular concentrations in hepatocytes could not be determined.

Dairy cows do not consume enough nutrients during parturition to cover the increased demand necessary to support lactation, leading to NEB; cows then start to mobilize body mass (Bell, 1995), and plasma NEFA, TG, and BHB levels increase. Similar results were obtained in the present study

and are schematically summarized for certain energy-related parameters (Fig. 2). We found differences in the TG and NEFA levels between the groups and, thus, in the curve shapes. Dietary LC supplementation resulted in higher serum TG concentrations in the CAR group before calving (Manuscript I), which may indicate a lower TG accumulation in the liver. The potentially lower NEFA levels during parturition in CAR cows in the current study (Manuscript I) might indicate increased transport of NEFA into the cells and subsequently into the mitochondrial matrix (Erfle et al., 1971). To improve the energy supply during the transition phase, dairy cows mobilize adipose tissue, providing TG and NEFA as energy sources for tissues and cells. Furthermore, ketone bodies, such as BHB, which are produced when the capacity of the carboxylic acid cycle is exceeded, can also be used as an energy source. On the one hand, lower NEFA values in the blood of CAR animals indicate better energetic utilization of NEFA by the cells of these animals and, on the other hand, better conversion of surplus NEFA to TG. Plasma concentrations reflect the results of production and utilization, and it is impossible to distinguish between the two processes that determine plasma levels. The ultrasonic data of adipose tissues suggested that LC did not affect energy metabolism or lipomobilization (Manuscript I). Numerically, however, it was shown that CAR cows were able to release more (0.40 MJ/MJ NEB) energy from adipose tissue for one MJ NEB than cows in the CON group (0.15 MJ/MJ NEB) (Manuscript I). This was estimated from the regression of energy released daily and the net energy balance (Manuscript I).

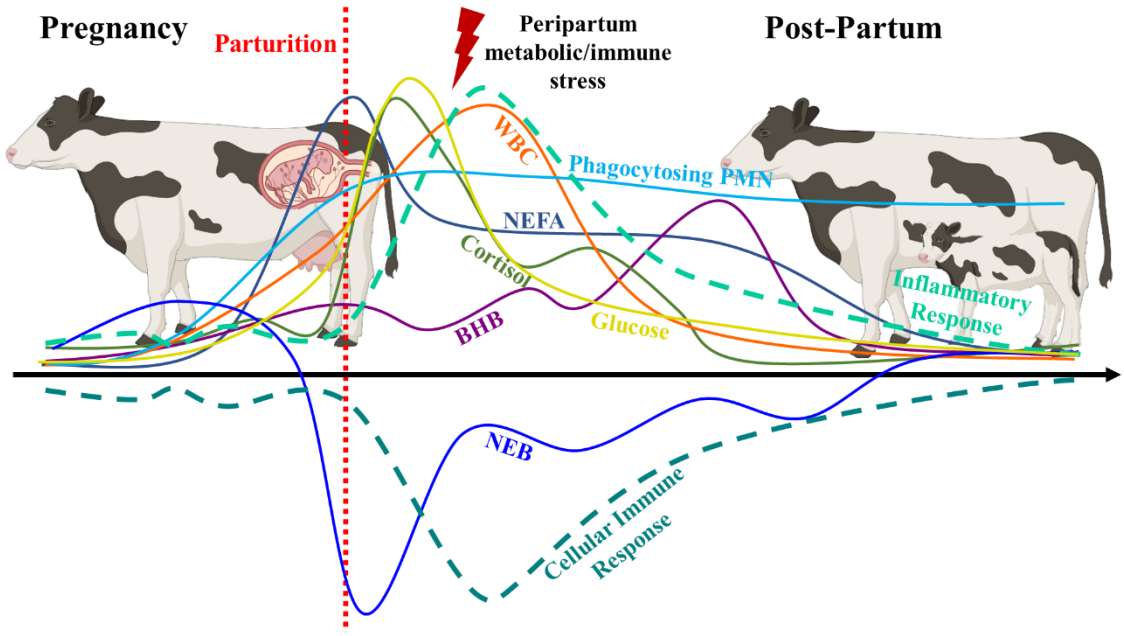

Figure 2: Schematic representation of the time courses data of selected parameters around calving. The results of the preceding study were used as a basis for the graphs. Inflammatory response and cellular immune response curves were adapted from Vlasova and Saif (2021). WBC = white blood cells; PMN = polymorph nuclear leukocytes; NEFA = non-esterified fatty acids; BHB = β hydroxybutyrate; NEB = negative energy balance.

Immune cells use NEFA and BHB as energy sources. Glucose is the main energy source for activated immune cells. The activated immune system of a cow requires 2.7 kg of glucose per day

(Kvidera et al., 2017). Before calving, DMI and, consequently, blood glucose levels decrease (Grummer, 1993) because of inadequate glucose utilization around parturition (Komatsu et al., 2005). In the current study, the highest blood glucose levels were measured shortly after calving (Manuscript I; Fig. 2). This coincided with the most critical period for immune cells, with a decrease in the cellular immune response and an increase in the inflammatory response (Fig. 2). However, owing to the rise and peak in glucose levels, it can be assumed that the supply of cells was sufficient. Nevertheless, it is suspected that the altered energy situation around calving substantially influences the cellular immune response. Reduced feed intake before parturition plays an important role in impairing peripartum immunity (Rukkwamsuk et al., 1999; Bertoni et al., 2009). Immunosuppression, independent of primary microbial or pathological infections, is often associated with parturition, referred to as inflammatory response in Figure 2. However, uterine and vaginal tissues are injured during birth (Sheldon and Dobson, 2004), which triggers inflammatory reactions by recruitment of peripheral immune cells and acute-phase reactions, as well as repair and remodeling processes. By dystocia-induced injury to the uterus, damage-associated molecular patterns (DAMP) are released from dying or necrotic cells or by extracellular matrix components (Healy et al., 2014). The released DAMP stimulates inflammatory events and binds to TLR (Tian et al., 2007). Healy et al. (2014) showed that animals with dystocia had increased concentrations of inflammatory mediators in the vaginal mucus 3 weeks *pp*. In addition, microorganisms and bacteria can penetrate injured tissues more easily and trigger inflammatory reactions in the mammary glands, uterus, and birth canal (Burvenich et al., 1999; LeBlanc, 2010).

Changes in immune cell populations, such as decreased lymphocytes, granulocytes, T-helper cells ($CD4^+$), cytotoxic T-cells ($CD8^+$), and altered immune cell functions (Menge and Dean-Nystrom, 2008; Oliveira et al., 2012) appearing around parturition are often interpreted as a reduced cellular immune response. This regularly observed immunosuppression can be aggravated by many factors, such as NEB, resulting in fat mobilization (Lacetera et al., 2005), oxidative stress (Sordillo et al., 2009b), ketosis, or altered hormone levels (Meglia et al., 2005). The weeks before and immediately after parturition are associated with neutrophilia, eosinopenia, lymphocytopenia, and monocytosis (Menge and Dean-Nystrom, 2008). This pattern of neutrophilia had previously been associated with physiological stress (Paglia and Valentine, 1967; Benzie and Strain, 1996) caused by an increase in cortisol (Belić et al., 2012; Schäfers et al., 2018). Elevated cortisol levels immediately after birth lead to the downregulation of L-selectin expression in bovine neutrophils, which, in turn, can inhibit the migration of neutrophil granulocytes into the tissue (Burton et al., 1995). L-selectin is involved in the adhesion of neutrophils to venous epithelial cells. Furthermore, elevated corticosteroid levels *pp* ensure that neutrophil granulocyte production increases in the bone marrow and that

demargination from the blood vessel wall occurs or a combination of both (Olmos et al., 2009; Tharwat et al., 2015).

Neutrophilia and lymphocytopenia were observed in the current study. Monocytosis, however, was not observed, whereas the proportion of peripheral eosinophils increased during the peripartum period (Manuscript III). In this study, blood cortisol levels also increased directly after parturition, as shown in Figure 2, indicating a higher stress level. To determine the extent to which granulocytes migrated or were newly formed in the bone marrow, the number of segmented and banded neutrophil granulocytes was assessed (Manuscript III). In our study, levels of segmented neutrophils, which correspond to mature neutrophils, increased continuously during late pregnancy (Manuscript III). A transient increase in immature neutrophil granulocytes (banded granulocytes) *pp* indicates a mild release of neutrophil granulocytes from the bone marrow. These findings confirm that the demarginalization of functional, mature neutrophils from the endothelium into the peripheral circulation may be most significant in the early postnatal period, thus explaining the increase in granulocyte counts. Additionally, eosinophil granulocytes in CAR cows significantly increased shortly before and after calving (Manuscript III). Together with the higher number of PLT in CAR cows than in CON cows (Manuscript II), this indicates that both cell populations might be mainly related to tissue repair and remodeling processes in direct response to parturition. On the one hand, this is in accordance with the already described evidence for tissue healing processes after calving (Hogan et al., 2008; Abdala-Valencia et al., 2018; Coden and Berdnikovs, 2020). The authors suggested that eosinophils and platelets influence the production of signaling molecules for the coagulation system mediated by eosinophils (Hogan et al., 2008; Abdala-Valencia et al., 2018; Coden and Berdnikovs, 2020). However, the influence of the LC on eosinophils at this level, as shown by our results, may indicate enhanced support for tissue repair and recovery after calving. In the last few years, it has been shown that eosinophils not only play a role in defense against pathogens but also influence tissue remodeling and the maintenance of homeostasis (Lee et al., 2010; Jacobsen et al., 2012).

Many studies have described the impaired capacity of neutrophils for phagocytosis (Kehrli, M. E., Jr. et al., 1989; Lacetera et al., 2005) or oxidative burst (Detilleux et al., 1995; Mehrzad et al., 2001) around parturition. In the present study, the proportion of phagocytosed PMN and the capacity of these cells to phagocytose, expressed as MFI, consistently increased during late pregnancy until calving (Fig. 2), whereas the LC-supplemented cows showed lower cellular uptake of bacteria around parturition (Manuscript III). Therefore, the current study describes a reduced capacity of PMN to phagocytose bacteria one week before to 9 h *pp* in CAR cows compared to CON cows. Almost simultaneously, these cells displayed significantly increased cellular efficiency in producing ROS after stimulation, as determined by the stimulation index (SI) of the MFI of

unstimulated and stimulated PMN. Although unstimulated intracellular ROS formation in PMN was significantly decreased in both groups during calving, LC supplementation tended to increase the proportion of ROS-producing PMN throughout the trial, revealing altered cellular activity in the CAR group. A higher SI of ROS forming PMN might indicate a temporarily improved efficiency of PMN around parturition due to LC supplementation. Additionally, significant correlations could be demonstrated between the functionality of PMN and PBMC *ex vivo* and parameters of energy metabolism *in vivo* (Manuscript III). Therefore, the data from the present study indicate an altered function of PMN during parturition and an improvement in the function of these cells by LC, thus contradicting the literature, which assumes a reduced cellular immune response (Fig. 2). However, our study also showed a decrease in the adaptive immune response of $CD4^+$ and $CD8^+$ cells and decreased proliferation of PBMC (Manuscript III). Nevertheless, it should be kept in mind that the proliferation of PBMC was only measured at 42 days *pp*. The number of B cells ($CD21^+$) increased before calving compared to that after calving (Manuscript III), indicating a shift in the immune response from Th1 to Th2.

Some studies have shown that plasma NEFA potentially interact directly with blood cells, resulting in changes in leukocyte function, inter alia, by modifying protein structure and function (Lacetera et al., 2005). Furthermore, concentration-dependent effects of NEFA on PMN viability and ROS production have been observed *in vitro* (Scalia et al., 2006). In fact, it is discussed that a reduced dry matter intake and increased NEFA concentrations lead to temporary immunosuppression in dairy cows (Kimura et al., 1999; Hammon et al., 2006). Reduced feed intake associated with NEB and increased NEFA concentrations was demonstrated in cows in the present study (Fig. 2, Manuscript I). Moreover, a correlation between *in vivo* NEFA and *in vitro* phagocytosis of PMN and the ROS stimulation index of PBMC was found (Manuscript III). Furthermore, LC supplementation increased the proportion of phagocytosed PMN in CAR cows after parturition. Because of their membrane-stabilizing properties (Pignatelli et al., 2003), LC prevents cell membrane damage due to oxidative stress (Saluk-Juszczak et al., 2010) and has been suggested to increase the protein-protein interactions of the cytoskeleton at the membranes of human erythrocytes, resulting in greater membrane stability (Allan Butterfield and Rangachari, 1993). Based on this and the results of the present study, it can be hypothesized that LC positively affects intracellular ROS production and phagocytic activity. During periods of physiological stress, such as parturition, cellular activity changes and the physiological state of the animals appear to play a significant role in the effect of LC supplementation. Many studies have reported the beneficial effects of LC on degenerative diseases and impaired membrane function (Cifone et al., 1997; Hurot et al., 2002; Bonomini et al., 2007; Lee et al., 2014; Mescka et al., 2015). Furthermore, LC supplementation improves and supports the physiological functions of neutrophil granulocytes in

aging rats (Izgüt-Uysal et al., 2003). It can be concluded that although all investigated cellular parameters were affected by parturition, no inhibitory effect on the proportion of phagocytosing cells, their ability to phagocytose bacteria, or the ability of NADPH oxidase to induce an oxidative burst was observed in the current study. This suggests that there might be taking place not directly as immunosuppression but as a change and adaptation around calving.

As mentioned in the introductory section (Section 2.3), calving leads to immunological changes and increased oxygen consumption by tissues and cells. Increased oxygen demand leads to increased ROS formation. If the antioxidative system cannot counteract this, oxidative stress occurs. This, in turn, affects the regulation of the immune system (Sordillo and Aitken, 2009a). Oxygen demand increases during pregnancy because of mammogenesis, lactogenesis, and galactogenesis (Akers, 2017). As a result of the increased oxygen demand, the blood flow and oxygen transport capacity increase. This is regulated by an increase in cardiac output and respiratory rate. In the present study, this increase was found for both parameters in the first hours after calving (Manuscript I). In addition, studies in cows and goats showed a positive correlation between blood flow and oxygen uptake in the chest as well as milk yield (Fleet and Peaker, 1978; Götze et al., 2010). With the onset of milk synthesis, both blood flow and oxygen uptake increase. An increase in oxygen uptake was also detected in this study owing to an increase in oxygen saturation (Manuscript I). Furthermore, the literature suggests the upregulation of erythropoiesis in the last trimester of pregnancy. The results of our study confirmed this, as all erythrocyte-related parameters, except for erythrocytes, showed increased values 42 days before parturition compared with 110 DIM and higher values immediately or shortly after calving, regardless of LC (Manuscript II).

Polycythemia and macrocytosis occur simultaneously with erythropoiesis, indicating an increase in the capacity of HGB to bind oxygen. This is shown by the increased proportion of oxyhemoglobin in the present study (Manuscript II). Oxygenated HGB tends to increase the rate of autoxidation (Abugo and Rifkind, 1994). This, in turn, increases the affinity of partially oxygenated HGB for erythrocyte membranes, resulting from autooxidation. This may lead to increased ROS formation at the membrane, resulting in peroxidative damage (Nagababu et al., 2008) and, consequently, compromised erythrocyte functionality. To protect cells from peroxidative damage, erythrocytes express enzymes that convert superoxide anions into hydrogen peroxide. In the present study, SOD and GPx activity was upregulated prior to calving (Manuscript II). After parturition, the levels of both enzymes declined rapidly (Manuscript II), which might indicate that late fetal development and parturition induced more pronounced erythrocytic oxidative stress than the time after parturition (Bernabucci et al., 2005). Similar results have been reported in other studies (Konvičná et al., 2015; Bühler et al., 2018). Additionally, nutritional antioxidants can be measured by plasma non-enzymatic antioxidative capacity (ferric reducing ability of plasma; FRAP) and derivatives of

reactive oxygen metabolites (dROM) (Manuscript II). As shown in the present study, FRAP and dROM increased within the first days *pp*, possibly due to improved feed intake compared to that before parturition (Manuscript I).

Taken together, the data collected in this study show that calving is a period in the production cycle of dairy cows that is associated with multilayered adaptation processes accompanied by metabolic and immunological stress (Fig. 2). This makes it difficult to examine individual parameters without considering other parameters. The immune system depends on many factors, such as hormones, energy metabolism, and oxidative stress, making single characterization difficult.

6 CONCLUSION

This study hypothesized that LC supplementation could positively influence the energy metabolism of cows and, thus, their cells, which in turn should positively influence hematology and cellular functions, particularly directly around calving.

It could be shown that there was a tendency for L-carnitine to change the level of NEFA and TG before calving. Furthermore, LC supplementation increased the number of PLT and eosinophils, and there was a positive effect on phagocytosis. However, it is important to mention that the influence of LC supplementation could only be shown for some parameters, and this was mostly only in the critical period directly around calving. An important issue that has to be considered, when using L-carnitine as a feed additive, is that the efficacy of supplemental L-carnitine is probably dependent on the animal's carnitine status, which is influenced by several factors: endogenous carnitine synthesis, carnitine uptake from the gastrointestinal tract and carnitine excretion (Ringseis et al., 2018). Unfortunately, the LC status of the animals could not be investigated further in this study.

Almost all the parameters investigated in this study showed a significant influence of time on parturition. These results indicated that the main alterations in the analyzed parameters occurred at the critical time directly before or after calving. These changes are probably induced by a metabolic challenge, as mentioned previously, as well as by stress, pain, physiological changes caused by parturition, and hormonal changes. Thus, in addition to the variables of energy metabolism (Manuscript I), the temporal progression of almost all analyzed parameters of the erythro- and leukograms, as well as platelets, illustrated adaptive processes before, at, and immediately after calving (Manuscript II, III).

In conclusion, based on the results of the present trial, it can be said that there was no immunosuppression in a strict sense, as described in the literature on calving. In contrast, it should be considered an adaptation of the immune response to changing circumstances. Furthermore, based on the data collected here, LC supplementation can only be recommended in the period directly around calving, as no improvements in various parameters could be achieved in the remaining period. However, it should also be taken into consideration that further studies should be conducted to determine the content of L-carnitine in the cell, liver, and kidney in order to obtain more accurate information about the L-carnitine status of the cow cells and to determine in which possibly not yet considered cases LC supplementation could have further positive effects on the cow and thus bring her better and healthier through the transit period.

7 SUMMARY

The transition period, defined as three weeks before to three weeks after parturition, is one of the most critical times in the production cycle of dairy cows. On the one hand, cows have to cope with increased energy demand, while on the other hand, feed intake decreases due to stress and pain during parturition. This results in an negative energy balance (NEB) and, consequently, at the beginning of body fat tissue mobilization (lipomobilization). Lipomobilization increases the blood concentration of non-esterified fatty acids (NEFA), because the liver cannot completely utilize these fatty acids. This is accompanied by an increase in the blood concentration of ketone bodies, such as β-hydroxybutyrate (BHB. In addition to changes in energy metabolism, alterations in the immune function of dairy cows occur during the transition period. Stress and pain during calving lead to elevated blood levels of glucocorticoids, such as cortisol, which affect the immune system. Furthermore, the immune system is affected by increased concentrations of NEFA and BHB. At the same time, oxidative stress occurs due to an imbalance between the production of reactive oxygen species (ROS) and the activity of the antioxidative system. In general, the period around calving and its consequences constitute a very complex process influenced by many interdependent factors. One key factor in energy production is the quaternary amine L-carnitine (LC), which is necessary for the transport of short-chain fatty acids from the cytosol to the mitochondrial matrix. Furthermore, several studies have demonstrated the antioxidant and membrane-stabilizing effects of LC.

This study aimed to investigate the effects of dietary LC supplementation on energy metabolism, hematology, and immune functions of dairy cows during the transition period. In addition, the first 72 h after calving were observed at high resolution to show the characteristic courses of the examined parameters, which, to the best of our knowledge, have not yet been analyzed.

To attain this aim, 60 pluriparous Holstein Friesian cows were assigned to two groups based on their lactation number, body weight, body condition score, and fat-corrected milk yield from previous lactation. The LC group (CAR) received 25 g of rumen-protected LC mixed with concentrate per cow per day. To compensate for the fat content of LC products required for rumen protection, the control group (CON) received an equivalent fat product. The study started 42 days before excepted calving and ended 110 days after parturition. Cows were fed a diet of 80% roughage and 20% concentrate until one day before calving. The concentrate was then increased from 30% to 50% until two weeks after parturition and maintained until the end of the trial.

To evaluate the performance and health of the animals, feed and milk samples were collected regularly, and feed intake, milk yield, body weight, and BCS were documented (Manuscript I).

Additionally, NEB was calculated, and NEFA, BHB, and triglyceride concentrations in the blood were determined (Manuscript I). Also, the concentration of LC in the blood as well as that of the precursors γ-butyrobetaine (γBB), Nε-trimethyllysine (TML), and acetylcarnitine (ACA) was examined (Manuscript I). Red blood cell counts and antioxidant enzyme activity were measured to obtain more information on the oxygen supply and antioxidant status of the animals (Manuscript II). To evaluate the immunological status and inflammatory response, white blood cell count, phagocytic activity, ROS production, and lymphocyte populations were analyzed (Manuscript III).

Dietary supplementation with LC increases blood LC, γBB, and ACA concentrations. Furthermore, LC supplementation resulted in better utilization of NEFA and TG. This was manifested by an increased blood concentration of triglycerides and a lower concentration of NEFA. Moreover, increased levels of platelets and eosinophils were detected in the CAR group, confirming the membrane-stabilizing effect of LC and the associated longer cell lifespan.

Additionally, immunological functions were affected by LC supplementation. The ability of polymorphonuclear cells (PMN) to phagocytose bacteria was analyzed by the mean fluorescence intensity (MFI) of ROS-producing PMN, and the phagocytic capacity decreased compared to the CON group. Simultaneously, the efficiency of ROS production by PMN increased in CAR cows. These results suggest an altered immune function around calving, but not suppression, as is often described in the literature.

In addition, this study showed that calving affected almost all analyzed data. The strongest changes in hematology and cell function were found four hours after calving. Furthermore, the influence of LC supplementation on immunological parameters was observed in the first few hours after parturition, indicating that LC supplementation may have an effect at energetically critical times.

In conclusion, the present study showed that dietary LC supplementation affected energy metabolism, cell vitality, and cell function during the critical period around calving. However, this study also showed the clear influences of calving, which may be even more pronounced than animal-specific differences. Future studies should record the LC supply of cells to enable a more detailed description of the energetic situation of cells such as blood cells.

8 ZUSAMMENFASSUNG

Die Übergangszeit, auch Transitphase genannt, ist eine der kritischsten Zeiten im Reproduktionszyklus einer Milchkuh. Zum einen hat die Kuh aufgrund des Einsetzens der Milchproduktion einen erhöhten Energiebedarf, zum anderen ist die Futteraufnahme durch die Geburt und den damit verbundenen Schmerz und Stress reduziert. Hieraus resultiert eine negative Energiebilanz (NEB) und der Körper beginnt Fettreserven abzubauen (Lipomobilisation), um ausreichend Energie bereitstellen zu können. Durch die Lipomobilisation erhöht sich die Konzentration an unveresterten Fettsäuren (NEFA) im Blut. Allerdings können diese Fettsäuren um die Geburt herum, aufgrund des Fehlens von Oxalsäure, nicht vollständig von der Leber zur Energiegewinnung über den Krebs-Zyklus verbraucht werden, sodass es zu erhöhten Konzentrationen von Ketonkörpern (BHB) im Blut kommt. Zusätzlich zu den erhöhten Belastungen des Energiestoffwechsels kommen in der Transitphase der Milchkuh massive Veränderungen in der Immunfunktion hinzu. Durch Stress und Schmerzen während der Geburt steigt der Blutspiegel der Glucocorticoide wie Cortisol, welche das Immunsystem beeinflussen. Darüber hinaus wird das Immunsystem durch die erhöhten Konzentrationen von NEFA und BHB beeinflusst. Gleichzeitig entsteht oxidativer Stress durch eine Imbalance in der Produktion von reaktiven Sauerstoffspezies (ROS) und der Aktivität des antioxidativen Systems. Insgesamt ist die Periode um die Kalbung ein sehr komplexer Prozess, welcher von vielen verschiedenen voneinander abhängigen Faktoren beeinflusst wird. Ein entscheidender Faktor ist hierbei die Energiebereitstellung und -gewinnung, um der negativen Energiebilanz entgegenzuwirken. Eine wichtige Schlüsselfunktion könnte dabei das quartäre Amin L-Carnitin (LC) einnehmen, das für den Transport von kurzkettigen Fettsäuren aus dem Zytosol in die mitochondriale Matrix notwendig ist. LC wird aus Lysin und Methionin in Gehirn, Leber und Niere synthetisiert und spielt eine wesentliche Rolle bei der Funktion der Mitochondrien, Energie bereitstellen zu können. Des Weiteren konnte in mehreren Studien eine antioxidative und membranstabilisierende Wirkung von LC nachgewiesen werden.

Die Hypothese dieser Arbeit war daher herauszufinden, ob eine LC Supplementierung einen positiven Einfluss auf den Energiestoffwechsel, die Hämatologie und die Immunfunktionen der Kuh während der Transitphase hat. Zusätzlich wurden, unserer Kenntnis nach zum ersten Mal, die ersten 72 Stunden nach der Kalbung hochaufgelöst untersucht, um charakteristische Verläufe einzelner untersuchter Parameter darstellen zu können.

Um diese Hypothese zu überprüfen, wurden 60 pluripare Holstein Friesian Kühe anhand ihrer Laktationsnummer, des Körpergewichts, dem Body Condition Score (BCS) und der fettkorrigierten Milchleistung der vorherigen Laktation in zwei Gruppen eingeteilt. Die L-Carnitin Gruppe (CAR)

erhielt 25 g pansengeschütztes LC pro Kuh und Tag, welches dem Kraftfutter beigemischt wurde. Um den Fettgehalt des LC-Produktes auszugleichen, welches für den Pansenschutz benötigt wurde, erhielt die Kontroll-Gruppe (CON) ein äquivalentes Fettprodukt. Der Versuch begann 42 Tage vor der erwarteten Kalbung und endete 110 Tag nach der Geburt. Die Kühe erhielten bis einen Tag vor der Kalbung ein Futter aus 80 % Raufutter und 20 % Kraftfutter. Der Kraftfutteranteil wurde dann bis zwei Wochen nach der Geburt von 30 % auf 50 % erhöht und bis zum Ende des Versuchs beibehalten.

Zur Bewertung der Produktionsleistung und der Gesundheit der Tiere wurden regelmäßig Futter- und Milchproben genommen, Futteraufnahme und Milchleistung erfasst sowie Körpergewicht und BCS festgehalten (Manuskript I). Um eine Aussage über den Energiestoffwechsel treffen zu können, wurde die NEB berechnet und den Tieren Blut entnommen, um die NEFA-, BHB- und Triglycerid-Konzentrationen zu bestimmen (Manuskript I). Ebenfalls wurde die LC- Konzentration im Blut untersucht sowie die der Vorläufer γ-Butyrobetain, Nε-Trimethyllysin und Acetylcarnitin (Manuskript I). Zusätzlich wurde das rote Blutbild und die Aktivität antioxidativer Enzyme bestimmt, um nähere Informationen zur Sauerstoffversorgung der Tiere und des antioxidativen Status zu erhalten (Manuskript II). Zur Bewertung von immunologischen Vorgängen und Entzündungsgeschehen wurde das weiße Blutbild aller Tiere analysiert sowie Phagozytoseaktivität, ROS-Produktion und Lymphozytenpopulationen näher untersucht (Manuskript III).

Durch die Supplementierung mit LC konnte ein Anstieg der LC-Blutspiegel sowie auch der Vorläuferstufen γBB und ACA festgestellt werden. Die LC Supplementierung führte zudem zu einer besseren Verwertung von NEFA und TG. Dies äußerte sich in einer erhöhten Konzentration von TG im Blut und einer niedrigeren NEFA-Konzentration. Außerdem konnten in der CAR Gruppe erhöhte Anteile von Thrombozyten und Eosinophilen festgestellt werden. Dies bestätigte die membranstabilisierende Wirkung von LC und eine damit verbundene längere Lebensdauer der Zellen.

Die Immunfunktion wurde ebenfalls durch LC beeinflusst. Die Fähigkeit der polymorph-nukleären Zellen (PMN) Bakterien aufzunehmen, wurde anhand der mittleren Fluoreszenzintensität (MFI) von ROS produzierenden PMN analysiert. Neben dieser Fähigkeit schien in der CAR Gruppe zur gleichen Zeit auch die Phagozytosekapazität geringer zu sein, begleitet von einer effizienteren Produktion von ROS durch die PMN. Diese Ergebnisse deuten zwar auf eine veränderte Immunfunktion um die Kalbung herum hin, allerdings nicht auf eine in der Literatur oft beschriebene Supprimierung.

Zusätzlich konnte gezeigt werden, dass die Kalbung nahezu alle erhobenen Daten beeinflusst hat. Die stärksten Veränderungen in der Hämatologie und der Zellfunktion wurden vier Stunden nach

der Kalbung festgestellt. In dieser Zeit wurden auch die Effekte durch LC gefunden, was darauf hindeutet, dass LC durchaus einen Einfluss haben kann, allerdings nicht generell, sondern nur zu energetisch kritischen Zeiten.

Abschließend konnte diese Studie zeigen, dass eine diätetische LC Supplementierung in kritischen Zeiten einen Einfluss auf den Energiestoffwechsel hat und die Vitalität und Funktion von Zellen beeinflussen kann. Allerdings zeigten sich in dieser Studie auch deutliche Einflüsse der Kalbung, welche möglicherweise sogar stärker ausgeprägt sind als tierindividuelle Unterschiede. Zukünftig sollte die Versorgung der Zellen mit LC differenziert erfasst werden, um eine genauere Aussage über die energetische Situation der Zellen treffen zu können. Zusätzlich sollten dabei auch interindividuelle Unterschiede der Tiere betrachtet werden.

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