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An Exploration of the Effects of an Early Postpartum Intravenous Infusion with Carnosic Acid on Physiological Responses of Transition Dairy Cows

Tainara Michelotti
South Dakota State University, tainara.michelotti@sdstate.edu

AN EXPLORATION OF THE EFFECTS OF AN EARLY POSTPARTUM INTRAVENOUS INFUSION WITH CARNOSIC ACID ON PHYSIOLOGICAL RESPONSES OF TRANSITION DAIRY COWS

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TAINARA MICHELOTTI

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This thesis is approved as a creditable and independent investigation by a candidate for the master's degree and is acceptable for meeting the thesis requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

Johan Osorio
Advisor Date

Joseph P Cassady
Department Head Date

Nicole Lounsbery, PhD
Director, Graduate School Date

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To my family, my friends, and all the research cows involved in this project.

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LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
BCS	Body condition score
BHB	Beta hydroxybutyrate
BW	Body weight
d	Days
DIM	Days in milk
DM	Dry matter
DMI	Dry matter intake
EB	Energy balance
ECM	Energy-corrected milk
Ex/Em	Fluorescence excitation/emission
IL-1	Interleukin 1
IL-6	Interleukin 6
NEB	Negative energy balance
NEFA	Non-esterified fatty acids
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B
PMNL	Polymorphonuclear neutrophils
ROS	Reactive oxygen species
SCC	Somatic count cell
TAG	Triacylglycerol
TMR	Total mix ration
TNFα	Tumor necrosis factor alpha
VFA	Volatile fatty acids
VLDL	Very-low density lipoprotein
wk	Week

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ABSTRACT

AN EXPLORATION OF THE EFFECTS OF AN EARLY POSTPARTUM INTRAVENOUS INFUSION WITH CARNOSIC ACID ON PHYSIOLOGICAL RESPONSES OF TRANSITION DAIRY COWS

TAINARA MICHELOTTI

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The transition period is commonly defined as the last 3 wk before parturition to 3 wk after parturition. It is the most critical stage of the lactation cycle of high-producing dairy cows. The period consists of a complex interplay of metabolic and hormonal adaptations, inflammation, and immune activation. During the transition period, dairy cows commonly face oxidative stress, an underlying factor for dysfunctional immune response and enhanced inflammation, which can further increase the susceptibility of dairy cattle to health disorders and decrease productivity. In the last few decades, there has been an increased interest in studying alternative natural compounds with antioxidant and anti-inflammatory properties as supplements for food-producing animals such as dairy cows. The aim of this project was to evaluate the effects of carnosic acid, a phenolic diterpene found in herbs from the Labiatae family, known for its antioxidant and antimicrobial properties, on both *in vivo* and *in vitro* models. The first objective was to evaluate the supplementation of carnosic acid on performance parameters and blood biomarkers of

transition dairy cows. The findings of this study revealed that carnosic acid promoted positive responses on inflammation and expression of oxidative stress biomarkers during the period of infusions. In addition, carnosic acid tended to increase lactation performance (e.g., milk yield and energy corrected milk), although the mechanisms responsible for this response remain unclear. The second objective was to evaluate the protective effects of carnosic acid on cultured mouse hepatocytes in response to oxidative challenge. This *in vitro* study revealed that carnosic acid promoted increased viability and antioxidant capacity through superoxide dismutase activity of mouse hepatocytes subjected to hydrogen peroxide-induced oxidative cell damage. In summary, the presented results reveal potential benefits of providing antioxidants such as carnosic acid during periods of oxidative challenge, such as the peripartal period of dairy cows.

CHAPTER 1. LITERATURE REVIEW

Transition period

The transition period, also called the periparturient period, is commonly defined as the last 3 wk before parturition to 3 wk after parturition (Grummer, 1995; Drackley, 1999) and is the most critical stage of the lactation cycle of high-producing dairy cows. The period consists of a complex interplay of metabolic and hormonal adaptations, inflammation, and immune activation (Pascottini et al., 2020).

Approximately 75% of the health disorders of lactating dairy cows (e. g., metritis, ketosis, lipidosis) typically happen in the first month after calving (LeBlanc et al., 2006). The high susceptibility to these diseases is mainly correlated with an impaired immune response as a result of drastic metabolic and endocrine changes during the transition period. Moreover, many of the metabolic disorders afflicting cows in the early post-partum period are interrelated and are associated with diet and management during the prepartum period (Overton and Waldron, 2004).

Despite numerous studies on the nutrition and physiology of transition dairy cows, this period remains a critical area for improvement on many dairy farms, with health disorders continuing to occur and causing a negative economic impact on the dairy industry. In fact, for decades, researchers, veterinarians, and nutritionists have tried to find solutions to overcome the challenges of maintaining health and welfare during the transition from late pregnancy to early lactation of high-producing dairy cows. Metabolic conditions in early lactation are responsible for direct profit loss due to the costs associated with disorders in clinical and subclinical forms and death of animals.

Furthermore, for animals that recover from diseases in early lactation, their productive potential over the entire lactation is likely compromised.

Physiology and metabolism

Negative energy balance

One of the main characteristics of the transition period is the drastic change in nutrient demands, which requires metabolism coordination to meet the new requirements for energy, glucose, amino acids, and calcium, to name a few (Overton and Waldron, 2004). The increase in nutrient requirements is mainly driven by fetal growth during the prepartum period and by lactogenesis after calving. Interestingly, prepartum dairy cows face a concomitant decrease in DMI prior to parturition, mainly related to physical, behavioral, metabolic, and hormonal changes (Contreras and Sordillo, 2011). A dramatic decline (around 20 to 40%) in DMI is commonly observed, particularly in the last week of gestation, followed by a slow rate of increase in DMI after calving (Hayirli and Grummer, 2004).

After parturition, however, the dietary intake is unable to meet the intense energy requirements, especially of glucose associated with the onset of lactation (Esposito et al., 2014; Lopreiato et al., 2020). The imbalance between the energy consumed and energy needed is termed negative energy balance (NEB) (Sordillo and Raphael, 2013). In high-producing dairy cows, the NEB is further aggravated by nutrient prioritization towards the mammary gland. In fact, after parturition, the energy demand for maintenance and milk production in high-yielding dairy cows can reach three times the amount necessary before parturition (Drackley et al., 2001; Weber et al., 2013). In this stage of reduced

nutrient and energy availability, dairy cows adapt to the NEB by mobilizing body reserves of different tissues, in particular fat tissue, and by turning skeletal muscle into a site of use of fat-derived fuels, allowing glucose to be redirected to lactogenesis (Contreras and Sordillo, 2011; Weber et al., 2013).

Lipid mobilization

In the adipose tissue, lipids are stored mainly as triacylglycerols (TAG), neutral lipids containing 3 fatty acids esterified to the carbon backbone of a glycerol molecule (Bolsoni-Lopes and Alonso-Vale, 2015). Lipid mobilization is a physiological adaptation by mammals to times of reduced nutrient and energy availability, characterized by an imbalance between lipogenesis (biosynthesis, incorporation, and storage of TAG in the adipocytes) and lipolysis (sequential hydrolysis of TAG, fatty acids, and glycerol) in the adipose tissue (Contreras and Sordillo, 2011).

In periods of high energy demands, as the onset of lactation, the adipocyte is stimulated by hormones, mainly catecholamines, to increase lipolysis at the expense of lipogenesis. The following is a series of intracellular reactions that culminate in the activation of hormone-sensitive lipase (HSL) and adipose triglyceride lipase (ATGL), enzymes responsible for about 95% of TAG hydrolysis (Bolsoni-Lopes and Alonso-Vale, 2015). From TAG hydrolysis, fatty acids are mainly released in the bloodstream bound to albumins as non-esterified fatty acids (NEFA) and used as an energy source by different tissues. At the same time, glycerol predominantly functions as a substrate for liver gluconeogenesis (Nelson, 2005).

During the transition period, lipidic mobilization is favored by a series of changes, including reduced levels of plasma insulin and glucose, reduced insulin sensitivity in adipose and other peripheral tissues, and increased levels of plasma catecholamines, growth hormone (GH), and glucocorticoids (Contreras and Sordillo, 2011; Humer et al., 2016). In the case of moderate lipid mobilization, plasma NEFA are used as an energy source, which helps conserve glucose for milk synthesis and consequently increases insulin concentrations, initiating a negative feedback to inhibit lipolysis.

However, when considering a high-yielding transition dairy cow, the intense lipid mobilization and excessive plasma NEFA concentrations are thought to disrupt this negative feedback mechanism. The high levels of plasma NEFA induce the expression of tumor necrosis factor α (TNFα), a cytokine that interferes with insulin-dependent reductions in lipase activity in the adipose tissue, further increasing the accumulation of plasma NEFA (Sordillo and Raphael, 2013). In periparturient dairy cows, the excessive post-partum body condition score reduction due to lipid mobilization and the consequent increase in plasma NEFA have been associated with risk of metabolic diseases related to intensive lipolysis (Contreras and Sordillo, 2011; McArt et al., 2013; Humer et al., 2016), herd removal, reproductive difficulty, and reduced milk production (McArt et al., 2013).

Non-esterified fatty acids metabolism

Once released from the adipose tissue, NEFA circulate throughout the body in the blood and be used as fuel by various tissues, such as muscle, mammary gland (for milk fat synthesis), or taken up by the liver (McArt et al., 2013). The liver is capable of removing around 15 to 20% of plasma NEFA, where they can be completely oxidized to

generate energy for the liver, partially oxidized to produce ketone bodies, or re-esterified into TAG, then either exported as very low-density lipoproteins (VLDL) or stored in the liver as such (Ospina et al., 2010; McArt et al., 2013). The ketone bodies released by the liver (acetone, acetoacetic acid, and mainly beta-hydroxybutyric acid [BHB]) can be used as an additional energy source by tissues such as the brain and heart (McArt et al., 2013).

In the periparturient dairy cow, plasma NEFA starts to rise 2 to 3 wk before calving, and peaks around calving or at the first week post-partum (Ingvartsen and Andersen, 2000), and as the concentration of NEFA in the blood increases, so does their uptake by hepatic tissue (Busato and Bionaz, 2020). The higher rates of NEFA entering the liver during the transition period sometimes exceed the organ's capacity to completely oxidize TAG or secrete TAG as VLDL, resulting in an accumulation of TAG in the liver and a greater production of ketone bodies by partial NEFA oxidation (Ospina et al., 2010; Gross et al., 2013).

In early lactation, a moderate increase in the concentration of NEFA and BHB in the blood is considered to be a normal adaptation to NEB. However, excessive concentrations of these metabolites are an indication of a poor adaptive response to NEB and intense lipid mobilization, and numerous studies have reported on the detrimental effects of elevated NEFA and BHB on early lactation immune response, milk production, and health events (McArt et al., 2013; McArt et al., 2015). During late lactation and most of the dry period, dairy cows have a positive energy balance and plasma NEFA average less than 0.2 mmol/L (Drackley, 2000; Contreras and Sordillo, 2011). The NEFA levels start to increase 2 wk before parturition, and between calving and the first week of lactation, NEFA can reach concentrations around 0.8 to 1.2 mmol/L, but should sharply

decrease as lactation progresses (Drackley, 2000). Nevertheless, transition dairy cows on a high lipid mobilization state are routinely exposed to circulation NEFA greater than 1.0 mmol/L, indicating a severe NEB (Drackley, 1999; Adewuyi et al., 2005; Busato and Bionaz, 2020).

Metabolic disorders of transition dairy cows

Fatty liver syndrome

The severe lipid mobilization often observed in early lactation of transition dairy cows is correlated with the development of fatty liver syndrome, also known as hepatic lipidosis. Hepatic lipidosis develops when the lipid influx to the liver exceeds the organ's capacity for NEFA oxidation or VLDL synthesis (Gross et al., 2013). Fatty liver primarily occurs in the first month after lactation, when up to 50% of cows would have some degree of TAG accumulation in the liver (Jorritsma et al., 2000).

Although the NEFA concentration at which TAG starts to accumulate in the liver is not well-established (Cooke et al., 2007), fatty liver syndrome is most likely to develop during periods of elevated plasma NEFA concentrations because rates of TAG synthesis are proportional to the NEFA supply to the hepatic tissue (Grummer, 1993). However, the direct evaluation of fatty liver is assessed by chemical or histological analysis of TAG within the liver tissue. Generally, fatty liver is categorized as normal, mild, moderate, or severe when liver TAG as % of wet liver weight is <1%, 1-5%, 5-10%, and >10%, respectively (Bobe et al., 2004). Depending on the level of histological and compositional alterations of the hepatocytes, the incidence of this syndrome is associated with reduced

metabolic function, health status, production, reproduction, and incidence of metabolic disorders and infectious diseases (Bobe et al., 2004; Gross et al., 2013).

Nutritional risk factors for fatty liver development are related to increased lipolysis or alterations in liver metabolism. The most common risk factors in the prepartum period are obesity (BCS $\geq$ 4.0), feed restriction, excess energy diets, and long calving intervals; while in the post-partum period, the occurrence of diseases and infections, fasting, feed restrictions, and sudden feed changes are correlated with fatty liver disease (Bobe et al., 2004; Kirovski and Sladojevic, 2017). In addition, Bobe et al. (2004) indicate that environmental stressors such as heat stress, overcrowding, and excessive pen movements, contribute to fatty liver development, mainly through decreasing dry matter intake.

Regarding the nutritional and management approaches for prevention of fatty liver, along with adequate control of the cited risk factors, Grummer (2008) stated that strategies could be subdivided as: (1) reducing lipolysis in adipose tissue; (2) increasing complete NEFA oxidation; and (3) increasing VLDL export from the liver. In this context, nutritional practices have been applied to prevent or treat fatty liver, especially by increasing VLDL secretion, since this mechanism is very limited in ruminants compared with other species (Kirovski and Sladojevic, 2017). Supplementation of substrates for the synthesis of VLDL such as choline increased VLDL secretion and alleviated TAG accumulation in the liver of dairy cows (Cooke et al., 2007; Coleman et al., 2019).

Ketosis

Ketosis is a metabolic disorder of common occurrence during early lactation, developed as a consequence of poor adaptive response to NEB and lipid mobilization (McArt et al., 2012). In the hepatic tissue, NEFA are broken down via β-oxidation into acetyl coenzyme A (acetyl-CoA). Acetyl-CoA then undergoes complete oxidation through the citric acid cycle to generate energy as ATP. However, during intense lipidic mobilization, increased levels of acetyl-CoA lead to an overload of the citric acid cycle, with depletion of its intermediates, resulting in increased ketogenesis, with the production of acetoacetic acid, acetone, and BHB. The synthesis of ketone bodies beyond the capacity of extrahepatic tissues to oxidize them leads to an increased concentration in body fluids, especially blood, where ketone bodies can lower pH and cause metabolic acidosis (Nelson, 2005).

Hyperketonemia has been defined as blood BHB concentrations above 1.4 µmol/L by some authors (e.g., Oetzel, 2004) and as concentrations above 1.2 µmol/L by others (e.g., McArt et al., 2012). Hyperketonemia can be manifested clinically as a drastic decrease in feed intake, body weight, and milk production (clinical ketosis, CK), but most likely as subclinical ketosis (SCK), with elevated blood ketone bodies without clinical signs (Oetzel, 2004; McArt et al., 2012). In addition to the direct profit loss associated with impaired milk production and treatment costs, elevated blood concentrations of ketone bodies have also been correlated with decreased reproduction performance and increased probability of other health disorders such as displaced abomasum and mastitis (Steeneveld et al., 2020).

Ketosis is mainly associated with early lactation of high-yield cows, with peak prevalence around the first week of lactation (McArt et al., 2012) and with more than 90% of SCK occurring in the first and second months post-partum (Rutherford et al., 2016). Although its incidence is widely variable among literature, depending on the diagnostic method and frequency of screening, consensus is that SCK has a higher incidence than CK. Overall, the incidence of CK in the first month of lactation is expected to be around 2 to 15%, whereas for SCK, the incidence can reach values greater than 40% during the same period (McArt et al., 2012; Gordon et al., 2013; Rutherford et al., 2016).

Immune system

The immune system can be defined as an interactive system of cells, tissues, and their soluble products that recognize, attack, and destroy pathogens that could endanger the homeostatic status of an organism (Mak et al., 2014a). The mammalian immune system can be divided into innate and acquired (also called adaptive) immune systems. These two immune systems are interconnected (Parkin and Cohen, 2001). While the innate immune system has a broad scope with the capacity to respond to tissue injury or neutralize a variety of potential pathogens, the adaptive immune system is a more specific response to infectious pathogens and can be augmented by repeated exposure to the same pathogen (Sordillo, 2016).

Innate immune system

Innate immune defenses in mammals are present in virtually all tissues. The innate immune response is typically rapid, and in contrast with the adaptive immune

system, traditionally described as lacking memory (Gasteiger et al., 2017). The main components of the innate immune system are physical barriers, pattern recognition receptors, complement, cytokines, and cellular components, which include endothelial, neutrophils, macrophages, monocytes, dendritic, and natural killer cells (Sordillo, 2016).

The very first line of defense against the entry of microorganisms and toxic molecules into the body are the physical barriers (mechanical, chemical, and biological). The mechanical barriers include the epidermis and keratinocytes of the skin and the GI and respiratory tract epithelium. The chemical barriers constitute pH secretions, and microbicidal molecules secreted from different tissues (e.g., skin, respiratory and gastrointestinal tract). In addition, commensal microbes in the skin and GI tract exist in symbiosis with the body and inhibit pathogens' growth (Doan, 2013).

When invading pathogens successfully break the mechanical barriers defenses, pattern recognition receptors (PRR) play a critical role in sensing their presence (Sordillo, 2016). The PRR are protein germline-encoded receptors capable of recognizing molecules frequently found in pathogens [called Pathogen-Associated Molecular Patterns (PAMPs)], or molecules released by damaged cells [called Damage-Associated Molecular Patterns (DAMPs)] (Gasteiger et al., 2017; Amarante-Mendes et al., 2018). Some examples of PAMP that can be recognized by PRRs include lipopeptides of gram-positive bacteria and LPS of gram-negative bacteria (Sordillo, 2016). Upon PAMP and DAMP sensing, PRR signal to the host the presence of infection and trigger proinflammatory and antimicrobial responses by activation of gene expression and synthesis of a series of molecules, including cytokines, cell adhesion molecules, and immunoreceptors (Mogensen, 2009; Kumar et al., 2011).

Cytokines are chemicals mainly secreted by immune cells and are one of the most important soluble defense mechanisms of the innate immune system. Cytokines act in a non-specific manner and are involved in a great variety of biological functions. For example, interleukin-1 (IL-1) is a cytokine that acts in the vascular endothelium, increasing its permeability to immune cells. Interleukin-8 (IL-8) also acts on the vascular endothelium, and its function is related to vascular endothelium activation and neutrophil attraction and activation (Doan, 2013).

In addition to the physical barriers and soluble components, the innate immune system includes cellular defenses, essential for an effective response against pathogens. Cellular defenses include neutrophils, a type of polymorphonuclear leukocytes (PMNL) (Doan, 2013; Sordillo, 2016). In response to local chemokine gradients, neutrophils are the first cells recruited to the site of inflammation. Once there, neutrophils are activated by binding their PRR to DAMPs or PAMPs. Neutrophils try to contain the foreign entities by phagocytosis, or when containment is not possible, they attempt to destroy pathogens by releasing destructive contents of their granules extracellularly, in a process called degranulation. Other cellular components include dendritic cells, monocytes, and macrophages, which are also phagocytic immune cells. Those cells also have the capacity to produce cytokines, which regulate or promote inflammation (Mak et al., 2014b).

Acute phase proteins

The acute phase proteins (APP) are blood proteins synthesized mainly by the liver and peripheral tissues (Ceciliani et al., 2012; Schneider, 2015). By definition, these proteins change their concentration by >25% in response to proinflammatory cytokines

stimulated during the onset of a disease (Eckersall and Bell, 2010). Acute phase proteins are believed to play major roles in the systemic reaction to inflammation, including opsonization, scavenging of toxic substances, and regulation of different stages of the inflammatory process (Ceciliani et al., 2012). Moreover, they are highly sensitive indicators of inflammation and can be used in disease diagnosis, prognosis, monitoring response to treatment, and general health screening (Eckersall and Bell, 2010).

Normal parturition in dairy cows is characterized by inflammatory-like conditions, during which proinflammatory cytokines, such as TNFα, IL-1, and IL-6, are produced in large amounts by immune cells. These cytokines can stimulate the liver to synthesize positive APP, such as haptoglobin or ceruloplasmin. In contrast, cytokines can impair the synthesis of negative APP (e.g., albumin and cholesterol), some of them important for normal liver metabolism (Bionaz et al., 2007). In cattle, haptoglobin and serum amyloid A (SAA) are considered major APP, meaning their concentration can increase up to and over 100-fold following inflammation (Thomas et al., 2015), whereas ceruloplasmin is classified as moderate or minor APP (Ceciliani et al., 2012).

Haptoglobin is considered a scavenger protein through its binding capacity. Haptoglobin eliminates metabolites such as free hemoglobin released from cellular degradation, helping to prevent oxidative tissue damage (Ceciliani et al., 2012; Moisa et al., 2019). Following parturition, high haptoglobin concentrations have been associated with leukocyte responses in dairy cows, including increased neutrophil surface expression of L-selectin and oxidative burst (Moisa et al., 2019). In contrast, ceruloplasmin is a copper carrier protein that oxidizes ferrous ions and has antioxidant and anti-inflammatory activity (Murata et al., 2004).

Periparturient immune dysfunction

The transition period is characterized by drastic physiological changes. Those changes include alterations in the immune system of the periparturient cow that ultimately lead to a reduction in immune competence and a concomitant systemic inflammatory response (Aleri et al., 2016; Trevisi and Minuti, 2018). It is clear that cows show an inflammatory condition from calving until around three weeks after parturition (Bertoni and Trevisi, 2013; Ingvartsen and Moyes, 2015; Trevisi and Minuti, 2018); changes in the immune system often proceed parturition (Kehrli et al., 1989a; b; Mezzetti et al., 2019). The prepartal immune alterations are correlated with a combination of endocrine and metabolic changes. However, it remains to be elucidated if the reduced immune competence is a physiological condition of transition dairy cows or an early signal of disease (Trevisi and Minuti, 2018).

Interestingly, the inflammatory state and positive acute phase mediators are present on the days following calving even in the absence of signs of microbial infections or other pathologies (Bionaz et al., 2007; Sordillo and Aitken, 2009; Bertoni and Trevisi, 2013). To some extent, the tissue damage associated with parturition and subsequent uterine involution and social stress of parturition contribute to the systemic inflammation of the transition cow (Bradford et al., 2015). Moreover, changes in the hormonal environment, especially the increase in glucocorticoids, have been associated with immunosuppression. However, these hormones are elevated for only 24 hours around parturition. Therefore, they could not alone explain the immune changes of the post-partum dairy cow, which remain for weeks following calving (Overton and Waldron, 2004; Ingvartsen and Moyes, 2015).

In addition to endocrine factors, several studies have correlated peripartal alterations in energy metabolism and nutrient supply with immunosuppression and immune dysfunction of dairy cows. Immune cells are affected by blood glucose levels, which is their preferred metabolic fuel rather than fatty acids, amino acids, or ketone bodies (Calder et al., 2007; Ingvartsen and Moyes, 2015). Additionally, glucose stimulates leukocyte immune response, including increased proliferation, differentiation, survival, chemotaxis, and phagocytosis (Ingvartsen and Moyes, 2013; Ingvartsen and Moyes, 2015). During early lactation, due to increased nutrient demands by the mammary gland, serum concentrations of glucose reach its nadir, and low glucose availability may limit immune function and increase risk of infection (Ingvartsen and Moyes, 2013). Furthermore, the lower blood calcium around parturition negatively impacts neutrophil activity. Intracellular calcium signaling is key in neutrophil activation and function (i.e., phagocytosis). Therefore, hypocalcemia around calving may also contribute to impaired immune response and greater susceptibility to diseases around calving (Ducusin et al., 2003; LeBlanc, 2020).

The low concentrations of glucose and calcium following parturition are often accompanied by increased levels of blood BHB and NEFA. In contrast with glucose, both BHB and NEFA have immunosuppressive effects and increase inflammation around parturition. Elevated levels of NEFA have been associated with impaired lymphocyte function (Lacetera et al., 2005; Ster et al., 2012), reduction in PMNL viability and killing ability, and increased neutrophils ROS production (Hammon et al., 2006; Scalia et al., 2006). In addition, NEFA has been demonstrated to enhance the proinflammatory phenotype of endothelial cells and to activate NF-κB signaling pathways, which can

induce the expression of several adhesion molecules, proinflammatory cytokines, and chemokines (Contreras and Sordillo, 2011; Contreras et al., 2012). Elevated concentrations of BHB decrease neutrophil phagocytosis, extracellular trap formation (Grinberg et al., 2008), and chemotaxis (Suriyasathaporn et al., 1999).

Oxidative stress

Oxidative stress can be defined as the damage occurring to cellular macromolecules as a consequence of a serious imbalance between oxidants and antioxidants (Halliwell, 2007; Mavangira and Sordillo, 2018). Oxidative damage has negative effects, especially on lipids, proteins, and DNA, and has been associated with the development of several pathologies. Reactive oxygen species (ROS) are the most abundant oxidants. However, reactive nitrogen species (RNS) also contribute to the overall oxidant pool (Mavangira and Sordillo, 2018). Commonly, RNS (e.g., nitroxide, peroxy-nitrite, and nitrogen dioxide) have been ascribed to ROS (Abuelo et al., 2015).

Reactive oxygen species

Reactive oxygen species are used in literature as a general term to describe different chemically reactive oxygen radicals as well as non-radical derivatives of oxygen (Patel et al., 2018). They are a consequence of the requirement of molecular oxygen as an electron donor for efficient energy production in aerobic organisms. Reactive oxygen species are mostly formed as normal end products of cellular metabolism of the mitochondrial electron transport chain (Sordillo and Aitken, 2009). In fact, in actively respiring mitochondria, 0.1% to 4% of O_2 used in respiration forms ROS (Nelson, 2005).

Other than mitochondrial respiration, sources of ROS production include the processes of the enzymes nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase), xanthine oxidoreductase, and myeloperoxidase (Patel et al., 2018). In mammalian cells, the biological sources of ROS also include endoplasmic reticulum, peroxisomes, cytosol, plasma membrane, and extracellular space (Ozcan and Ogun, 2015).

Reactive oxygen species include the free radicals superoxide anion ($O_2^{-}\bullet$), hydroxyl radical ($OH\bullet$), and hydroperoxyl ($HO_2\bullet$), and the non-radicals hydrogen peroxide (H_2O_2), hypochlorous acid ($HOCl^{-}$), and single oxygen ($^1O_2$) (Sordillo and Raphael, 2013). There are many enzymatic pathways in which ROS are produced in the cell, and most pathways are initiated by the production of superoxide (Patel et al., 2018). The superoxide anion is a highly reactive free radical produced mainly in the mitochondria, and its formation also leads to the production of a variety of other ROS, including hydroxyl free radical, which is even more reactive (Nelson, 2005; Sordillo and Aitken, 2009).

Reactive oxygen species physiological and pathological effects

Low to moderate concentrations of ROS are necessary for the regulation of physiological processes and redox homeostasis, such as cell differentiation and proliferation (Sordillo and Raphael, 2013; Abuelo et al., 2015). In the host immune response, ROS play an important role in the destruction of pathogens and the expression of immunoregulatory substances (Abuelo et al., 2015). In addition, according to Nelson

(2005), low levels of ROS may be used as an indicator of insufficient oxygen supply, triggering metabolic changes.

Immune phagocytes, such as macrophages, neutrophils, and dendritic cells, can produce ROS in their endosomes through NADPH oxidase activity. Following the engulfing of pathogens by phagocytosis, these immune cells degrade the invasive organism by releasing ROS from their cytoplasmic granules (Sordillo and Raphael, 2013; Patel et al., 2018). This mechanism is named oxidative burst and is one of the components of an effective first line of defense of the innate immune system (Pascottini et al., 2019).

Although ROS are essential physiological functions, their concentrations are finely regulated by several endogenous antioxidant defense mechanisms. In cases of redox imbalance, either by increased production of ROS or failure of the defense mechanisms, the consequent oxidative stress results in the occurrence of various deleterious processes that could ultimately lead to cell death. Among the most susceptible molecules to ROS damage is DNA. This damage has detrimental effects, including carcinogenesis, mutations, genome instability, and cell signaling alterations (Sordillo and Raphael, 2013; Surai et al., 2019).

The complex structure of proteins, aligned to the presence of oxidizable functional groups (e.g., thiol (─SH) found on cysteine residues), results in susceptibility to oxidative damage (Patel et al., 2018; Surai et al., 2019). Overall, protein oxidation can alter their functional structure and consequently compromising their biological activity. Moreover, lipids, especially the phospholipids present in the cell membrane, are

susceptible to peroxidation and the subsequent formation of lipid radicals (Sordillo and Raphael, 2013). These lipid radicals can stimulate the oxidation of adjacent fatty acids, entering a vicious cycle that can lead to membrane function loss and cell death (Abuelo et al., 2015).

Antioxidant mechanisms

Antioxidant defense systems are believed to have evolved as a means of surviving in an oxygenated atmosphere (Surai et al., 2019). Animal antioxidant mechanisms are based mainly on the presence of biological compounds named antioxidants, either biologically synthesized or provided in the diet (Surai et al., 2019). In general terms, antioxidants can be defined as any substance that delays, prevents, or removes oxidative damage to a target molecule (Halliwell, 2007).

The antioxidant network operates at both cellular and subcellular levels and is based on several lines of defense. These antioxidant strategies include direct scavenger of ROS, antioxidant recycling, metal binding and chelating, removal of damaged molecules, and decreased activity of pro-oxidant enzymes, among others (Surai et al., 2017; Surai et al., 2019). Antioxidant compounds can be further classified based on several methods, such as solubility in lipids or water. Most commonly, antioxidants are divided based on their chemical and physical characteristics as enzymatic or nonenzymatic (Sordillo and Aitken, 2009; Morillas-Ruiz and Hernández-Sánchez, 2015).

The main antioxidant enzymes are superoxide dismutase (SOD, which reduces superoxide anion), catalase (CAT, catalyzes the reduction of hydrogen peroxide), and glutathione peroxidase (GPx, catalyzes the degradation of hydrogen peroxide and organic

peroxides). Nonenzymatic antioxidants include glutathione, ascorbic acid (vitamin C), α-tocopherol (vitamin E), β-carotene (vitamin A), uric acid, polyphenols, and others (Sordillo and Raphael, 2013; Morillas-Ruiz and Hernández-Sánchez, 2015). Vitamin C is known as a radical scavenger, while vitamins E and A have a vital function in preventing and disrupting fatty acid peroxidation chain reaction, respectively (Sordillo, 2016).

Oxidative stress of transition dairy cows

During the transition period, dairy cows undergo a shift from a gestational nonlactating state to a state of copious milk synthesis and secretion, demanding metabolic adjustments to the dramatic increase in energy requirements (Sordillo and Raphael, 2013). This period of enhanced metabolism leads to a considerable increase in oxygen requirements and the consequent production of oxidants (Sordillo and Aitken, 2009; Abuelo et al., 2015; Abuelo et al., 2016a). In particular, the intensified processes of NEFA β-oxidation in the liver, characteristic of the negative energy balance during the transition period, results in the increased production of ROS (Turk et al., 2013). In addition to the increased ROS production in the liver, the proinflammatory state of the transition dairy cow also contributes to alterations in redox balance. This mechanism occurs through the release of cytokines, which can induce ROS production by both phagocytic and non-phagocytic cells (Thannickal and Fanburg, 2000; Valko et al., 2007).

Following parturition, ROS production often exceeds the antioxidant capacity of the tissues, leading to a progressive development of oxidative stress (Abuelo et al., 2015; Abuelo et al., 2016a). Oxidative stress can further increase dysfunctional inflammation and metabolic stress (Figure 1.1), creating a vicious cycle that ultimately leads to tissue

damage, increased risk of health disorders, and decreased milk production and reproduction of the transition dairy cow. In immune cells, alterations in redox balance can result in diminished functional activities, such as reduction in neutrophil phagocytosis and killing capacity (Sordillo and Aitken, 2009). In addition, oxidative stress has been correlated with increased inflammation by activating proinflammatory signaling pathways (Sordillo and Raphael, 2013) such as NF-κB, and increased expression of proinflammatory mediators (i.e., TNFα and IL-6). Furthermore, oxidative status has been associated with insulin resistance in periparturient dairy cattle, which, in turn, can further enhance the lipolytic state during the transition period (Abuelo et al., 2016b).

Overall, oxidative stress is an underlying factor for dysfunctional immune response and inflammation, which underscores the importance of this condition in increasing the susceptibility of dairy cattle to health disorders, particularly during the transition period (Bernabucci et al., 2005; Sordillo and Aitken, 2009; Abuelo et al., 2015). Therefore, during this period of high antioxidant demands, dietary supplementation with antioxidants is recommended to meet the increased requirements (NRC, 2001; Spears and Weiss, 2008; Abuelo et al., 2019).

In dairy cattle, vitamin E and selenium are the most widely used antioxidants included in diets, especially in the form of premixes added to the total mixed ration (Abuelo et al., 2015). Although the NRC (2001) acknowledged that additional vitamin E might be useful during periods of immunosuppression such as the peripartal period, several factors seem to influence the recommended values for antioxidants requirement. The supplementation of antioxidants has been mostly beneficial for animal health status

(Politis et al., 1995; Politis et al., 1996). However, recent studies have reported deleterious effects of excessive supplementation of antioxidants. For example, provisions of 3,000 IU of vitamin E, which exceeds NRC standards, increased the risk of mastitis and oxidative stress in the postpartum period of transition dairy cows (Bouwstra et al., 2010a; Bouwstra et al., 2010b).

Use of nutraceuticals during the transition period of dairy cows

For decades, researchers, veterinarians, and nutritionists in the dairy industry have been trying to overcome the challenges of maintaining health and welfare during the transition period of high-producing dairy cows. The complexity of the physiological changes around parturition, aligned with the direct effects of energy and nutrient status on immune function, makes this period particularly challenging, requiring the application of multiples strategies to allow a better transition into lactation. In general, the nutritional management strategies of transition dairy cows aim to meet glucose demands, decrease excessive lipid mobilization, reduce TAG accumulation in the liver, prevent hypocalcemia, and ultimately, reduce immune dysfunction and the incidence of health disorders (Overton and Waldron, 2004; Sordillo, 2016).

The dairy industry has recently faced an increased consumer preference for healthier and natural food sources, including concern for animal welfare and decreased use of antibiotics. In contrast to antimicrobial drugs used to treat diseases, strategies that target host responses through nutrition can minimize drug residues and the possibility of developing drug-resistant pathogens (Sordillo, 2016). There has been an increased interest in supplementing feed with alternative natural compounds with antimicrobial,

antiparasitic, and immunomodulation properties for food-producing animals (Yang et al., 2015; Ciampi et al., 2020; Lopreiato et al., 2020). These alternative compounds have become a focus for researchers and producers because their beneficial effects may be transferred to the final products for the human diet, which are positively perceived by consumers while maintaining a profitable margin for dairy farm operations (Abuelo et al., 2019; Bhagat et al., 2019).

These alternative natural compounds are often referred to as "nutraceuticals" as their health benefits reach beyond nutritional value. Nutraceuticals interact at different levels of the animal's physiology and are considered between the lines of feed and a drug (Yurdakok-Dikmen and Filazi, 2019; Lopreiato et al., 2020). Nutraceuticals are mostly multi-target polypharmacological agents, presenting antioxidants and anti-inflammatory properties, along with benefits in terms of cell survival, differentiation, and proliferation (Dormán et al., 2016; Lopreiato et al., 2020), making their supplementation particularly beneficial during the transition period of high-producing dairy cows. However, regardless of potential applications, due to their classification as "dietary supplements", these natural compounds lack governmental regulations and information regarding composition, dosages, effectiveness, and quality, which are not necessarily validated (Ballou et al., 2019).

Nutraceuticals can be classified in many ways based on their mechanism of action, chemical structure, and source, among others. In a recent review, Ballou et al. (2019) classified the nutraceuticals currently available for ruminants into: 1) probiotics, 2) prebiotics, 3) phytonutrients, or 4) polyunsaturated fatty acids. Probiotics confer beneficial health effects on the host mainly by improving the balance of the healthy

microflora (beneficial bacteria), enhancing mucosal immunity, increasing digestive capacity, and preventing colonization of harmful bacteria (Yurdakok-Dikmen and Filazi, 2019). A multi-strain probiotic supplement containing *Saccharomyces cerevisiae* and *Lactobacillus sporogenes* improved milk yield, fat corrected milk (FCM), and milk solids in early to mid-lactating dairy cows (Shreedhar et al., 2016). In addition, dairy cows supplemented with *Lactobacillus casei* and *Lactobacillus plantarum* from 60 to 90 DIM had increased milk production and lower SCC than control cows (Xu et al., 2017).

Prebiotics are defined as indigestible carbohydrates, such as oligosaccharides and fructans, that serve as an energy source for commensal or probiotic bacteria in the rumen and lower GIT (Ballou et al., 2019). Yeast culture products from *Saccharomyces cerevisiae* have improved energy corrected milk (ECM), decreased SCC, and increased abundance of cellulolytic and lactate-utilizing bacteria in transition dairy cows (Carpinelli et al., 2021) while promoting more neutral rumen pH and greater total VFA in lactating dairy cows (Halfen et al., 2021).

Another classification of nutraceuticals is phytonutrients, also known as phytochemicals, plant-derived bioactive compounds with various chemical structures and biological activities (Oh et al., 2017; Yurdakok-Dikmen and Filazi, 2019). Major phytonutrients used in farming systems include phenolic compounds, saponins, and essential oils (Vasta and Luciano, 2011; Yurdakok-Dikmen and Filazi, 2019). Phytonutrients have been investigated as feed additivities for their potential use as antioxidants, antimicrobials (Hammer et al., 1999), immune stimulators, and modulators of rumen fermentation (Wallace, 2004).

Polyphenol compounds represent a family of phytochemicals of about 500 different molecules with potential therapeutic applications due to their intrinsic antioxidative and anti-inflammatory properties (Ballou et al., 2019; Lopreiato et al., 2020). Some of the more common plants containing polyphenols include thyme and oregano, clove, juniper, cinnamon, hot peppers, garlic, aloe, yerba mate, pomegranate, silymarin, and green tea (Lopreiato et al., 2020). Mezzetti et al. (2020) reported positive effects on liver function and redox balance when transition dairy cows were supplemented with *Aloe arborescens*, while Ciampi et al. (2020) observed that pomegranate extract had the potential to mitigate oxidative stress without detrimental effects on bovine endothelial cell viability *in vitro.*

Carnosic acid

Herbs from the Labiatae family, especially common salvia (*Salvia officinalis*) and rosemary (*Salvia rosmarinus*), are known to have a particularly high concentration of phenolic diterpenes with antioxidant and antimicrobial properties, such as carnosic acid, carnosol, and rosmarinic acid (Birtic et al., 2015; Loussouarn et al., 2017). Among these phenolic diterpenes, carnosic acid is the most abundant in rosemary leaves, where they can reach values greater than 10 mg/g of fresh weight (Luis and Johnson, 2005), and it has one of the highest antioxidant activities (Cuvelier et al., 1994; Okamura et al., 1994).

Carnosic acid has a high reactivity toward ROS and acts as a scavenger that can eliminate ROS (Birtic et al., 2015). Moreover, carnosic acid plays a role in the activation of the PI3K/Akt/NRF2 signaling pathway in human cells (de Oliveira et al., 2015; de Oliveira et al., 2016). Nuclear factor erythroid-2 related factor 2 (NRF2) is a transcription factor involved in the cellular response to oxidative stress, inducing the expression of

several protective enzymes, such as glutathione peroxidase and superoxide dismutase (de Oliveira et al., 2016). Carnosic acid has also been correlated with reduced expression levels of proinflammatory cytokines, e.g., IL-1, IL-6, and TNFα in human and mice cells through the NF-κB signaling pathway (Liu et al., 2019; Hosokawa et al., 2020). Moreover, carnosic acid suppresses hepatic steatosis in high-fat diet-fed mice through the downregulation of hepatic genes related to fatty acid synthesis (e.g., fatty acid synthase) and upregulation of hepatic genes related to β-oxidation (e.g., PPARα, peroxisome proliferator-activated receptor-α) (Park and Mun, 2013).

A summary of recent studies supplementing rosemary extracts and leaves in ruminant species and their main findings can be found in Table 1. In ruminant species, studies evaluating carnosic acid and rosemary supplementation have been associated with an increase in meat quality and milk production of goats and sheep. Surprisingly, the strategy used by carnosic acid to reduce the oxidative and metabolic stress of transition dairy cows has not been explored until the present moment.

RATIONALE AND OBJECTIVES

The overall goal of this project was to explore the effects of carnosic acid on biological models of oxidative stress. Therefore, the objectives of this study were to evaluate the supplementation of carnosic acid on: a) performance parameters and blood biomarkers of transition dairy cows infused with carnosic acid; and b) protective effects of carnosic acid on cultured mouse hepatocytes in response to oxidative challenge. To the best of our knowledge, carnosic acid has never been studied as a nutraceutical in dairy or beef cattle, and it could represent a breakthrough in oxidative stress protection in transition dairy cows. Based on the anti-inflammatory and antioxidant properties of

carnosic acid, we hypothesized that carnosic acid would have cytoprotective effects in cultured mouse hepatocytes subjected to hydrogen peroxide-induced oxidative cell damage. Moreover, considering its chemical properties and previous studies using different animal models, we hypothesized that providing carnosic acid during early lactation might minimize the typical exposure to oxidative stress and attenuate the typical inflammatory response during the peripartum period while allowing cows to reach peak performance.

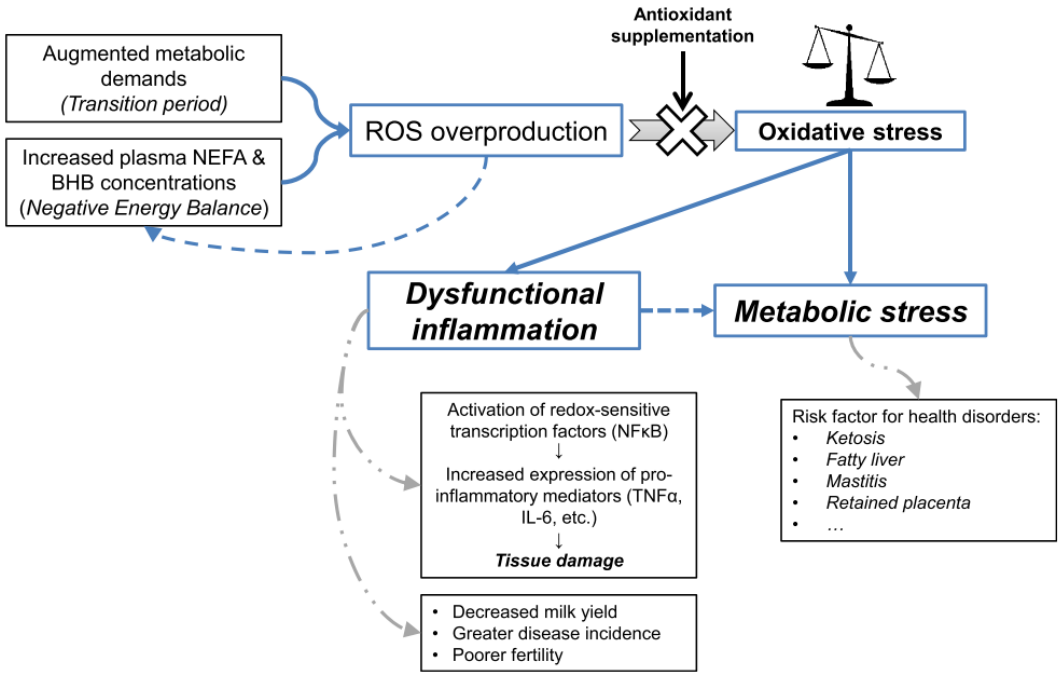

Figure 1.1. Schematic illustration of the interplay between antioxidant supplementation, metabolic stress, dysfunctional inflammation and presented health disorders.

Source: Abuelo et al. (2015).

Table 1.1. Studies with carnosic acid and rosemary supplementation in different ruminant species.

Subject	Supplement	Dose	Period of supplementation	Effect	Reference
22 lambs	Carnosic acid	0.6 g/kg of DM; 1.2 g/kg of DM	5 wks	Delayed lipid peroxidation in the meat.	Moran et al. (2012)
30 lambs	Rosemary extract	640 ppm of 1:1 CA: carnosol; 685 ppm of 2:1 CA: carnosol	56 d	Transfer of CA to meat; increased antioxidant status of meat.	Jordan et al. (2014)
36 sheep and 27 lambs	Rosemary leaves	10% or 20% of basal diet (pellet of 50% barley and 50% distilled rosemary leaves)	240 d	Lower lipid oxidation, higher colour stability	Nieto et al. (2010)
36 sheep	Rosemary extract	600 mg/head/d 1200 mg/head/d	0 to 154 DIM	Higher doses of rosemary extract increased milk yield, protein (%), casein (g), and lactose (% and g).	Chiofalo et al. (2010)
30 dairy goats	Rosemary leaves or extract	Rosemary leaves: 60 g/kg of DM Rosemary extract: 0.6 g/kg of DM	80 d	Greater milk, milk fat and milk protein production.	Smeti et al. (2015)
15 dairy goats	Rosemary leaves	10 g/head/d	12 wks	Greater nutrient digestibility, and ruminal fermentation. Increased milk yield and positive effects on milk fatty acid profile.	Kholif et al. (2017)
36 dairy goats	Rosemary leaves	10% or 20% of basal diet (pellet of 50% barley and 50% distilled rosemary leaves)	224 d	Alter neither yield nor milk quality. Increased concentration of polyphenols components in the milk and in the plasma of the suckling goat kid.	Jordan et al. (2010)

CHAPTER 2. AN EXPLORATION OF THE EFFECTS OF AN EARLY POSTPARTUM INTRAVENOUS INFUSION WITH CARNOSIC ACID ON PHYSIOLOGICAL RESPONSES OF TRANSITION DAIRY COWS

Abstract

The objective of the present study was to evaluate the effects of an antioxidant and anti-inflammatory compound found in rosemary plants (*Salvia rosmarinus*) named carnosic acid during the transition period of dairy cows. From day 1 to 3 after calving, 16 multiparous Holstein cows received a daily intravenous infusion of either 500 mL of saline (NaCl 0.9%; Saline; n = 8) or carnosic acid at a rate of 0.3 mg/kg of BW supplied in 500 mL of saline (CA; n = 8). Blood samples were taken at –7, 2, 5, 7, 14, and 21 d relative to parturition, then analyzed for metabolites related to energy metabolism, muscle mass catabolism, liver function, inflammation, and oxidative stress. Infusion of CA tended to improve milk performance; however, DMI was unaffected by treatment. At 2 d relative to parturition, CA cows had lower blood concentrations of haptoglobin, paraoxonase, FRAP, and NO_2^- than saline cows. After treatment infusions, haptoglobin remained lower in CA cows than saline at 5 d relative to parturition. Our results demonstrate that carnosic acid promoted positive responses on inflammation and oxidative stress biomarkers and may promote beneficial effects on lactation performance in peripartal dairy cows.

Keywords: peripartum; oxidative stress; carnosic acid

Introduction

The transition from pregnancy to lactation is one of the more stressful periods of a dairy cow's life. Approximately 75% of health disorders in adult cows, such as retained placenta, mastitis, and metritis, typically occur within the first month after calving (LeBlanc et al., 2006). The high susceptibility to these diseases is mainly correlated with an impaired immune response, a result of drastic metabolic and endocrine changes during the transition period.

From a metabolic standpoint, dairy cows commonly experience increased energy requirements during the transition period, primarily for fetal growth prepartum, followed by milk synthesis postpartum (Drackley, 1999; Sordillo and Aitken, 2009). Cows showed a clear inflammatory response (Bertoni and Trevisi, 2013; Trevisi and Minuti, 2018) after calving, although changes in immune response often proceeded parturition (Kehrli et al., 1989a; b; Mezzetti et al., 2019a). Additionally, during the prepartum period, cows commonly experienced a decrease in dry matter intake (DMI) (Hayirli and Grummer, 2004). Although immediately after parturition, there is a progressive increase in feed intake, which generally cannot meet nutrient requirements in early lactation. Therefore, a negative energy balance (NEB) condition is reached, and consequently, non-esterified fatty acids (NEFA) are mobilized from the adipose tissue to meet nutrient requirements (Contreras and Sordillo, 2011; Abuelo et al., 2015).

During NEB, triacylglycerides (TAG) are hydrolyzed into glycerol and NEFA and released into the bloodstream (Nelson, 2005). The NEFA can be used as an energy source by different tissues, such as the mammary gland for milk fat synthesis, or taken up by the liver (McArt et al., 2013). In the liver, NEFA are either completely oxidized to

generate energy, partially oxidized to produce ketone bodies, or re-esterified into TAG. Then, TAG are either exported as very low-density lipoproteins (VLDL) or stored in the hepatocytes (Ospina et al., 2010; McArt et al., 2013). At periods of intense lipid mobilization, like the transition period, NEFA entering the liver exceeds its capacity for β-oxidation and VLDL secretion, which leads to a greater production of ketone bodies, such as β-hydroxybutyrate (BHB), and the accumulation of TAG in the liver (Ospina et al., 2010; Gross et al., 2013).

The increased plasma NEFA and BHB, aligned with augmented metabolic demand characteristic of the transition period, is commonly accompanied by a surge in reactive oxygen species (ROS) leading to oxidative stress (Sordillo and Raphael, 2013; Abuelo et al., 2015). In parallel, the proinflammatory cytokines released by the immune system induce ROS production by both phagocytic and non-phagocytic cells (Abuelo et al., 2015), which can further alter redox balance and lead to an oxidative stress condition during early postpartum period. Oxidative stress is defined as the damage occurring to cellular macromolecules as a consequence of serious and prolonged imbalance between oxidants and antioxidants (Halliwell, 2007b; Mavangira and Sordillo, 2018).

The ROS are particularly reactive towards DNA, proteins, and lipids, resulting in cellular alterations, including lipid peroxidation, cell membrane damage, DNA mutation, impaired protein activity, and cell death (Sordillo and Raphael, 2013; Abuelo et al., 2016b; Mavangira and Sordillo, 2018). In immune cells, those alterations can result in diminished functional activities, such as reduction in neutrophil phagocytosis and killing capacity (Sordillo and Aitken, 2009). In addition, oxidative stress has been correlated with increased inflammation through the activation of proinflammatory signaling

pathways (Sordillo and Raphael, 2013) and with insulin resistance, which can further enhance the lipolytic state of transition dairy cows (Abuelo et al., 2016b). Taken together, increased oxidative stress is associated with a dysfunctional immune response and enhanced inflammation, resulting in increased susceptibility of dairy cattle to health disorders, particularly during the transition period (Bernabucci et al., 2005; Sordillo and Aitken, 2009; Abuelo et al., 2015).

Antioxidant compounds can be synthesized by the body and are also naturally present in feedstuffs; however, for indoor animals fed low forage diets or especially during periods of high antioxidant demands such as the peripartal period, dietary supplementation is necessary to meet increased requirements (NRC, 2001; Spears and Weiss, 2008; Abuelo et al., 2019). Vitamin E and selenium are the most widely used antioxidants supplemented in dairy cattle diets, especially in the form of premixes added to the total mixed ration (Abuelo et al., 2015). However, in the last few decades, there has been an increased interest in studying alternative natural compounds with antioxidant and anti-inflammatory properties as feed supplements for food-producing animals (Yang et al., 2015; Ciampi et al., 2020; Lopreiato et al., 2020). Besides being a potential source of antioxidants in food consumed by humans, these natural compounds have the added benefit of being positively perceived by consumers (Abuelo et al., 2019; Bhagat et al., 2019).

Rosemary (*Salvia rosmarinus*), an herb from the Labiatae family, is known to have a particularly high concentration of phenolic diterpenes with antioxidant and antimicrobial properties (Birtic et al., 2015). Among those phenolic diterpenes, carnosic acid is the most abundant in rosemary leaves (Luis and Johnson, 2005) and has one of the

highest antioxidant activities (Cuvelier et al., 1994; Okamura et al., 1994). Carnosic acid has high reactivity toward ROS and acts as a scavenger that can eliminate ROS (Birtic et al., 2015). Moreover, carnosic acid plays a role in the activation of the PI3K/Akt/Nrf2 signaling pathway in human cells (de Oliveira et al., 2015; de Oliveira et al., 2016). Nuclear factor erythroid-2 related factor 2 (Nrf2) is a transcription factor involved in the cellular response to oxidative stress, inducing the expression of several protective enzymes, e.g., glutathione peroxidase and superoxide dismutase (de Oliveira et al., 2016). Furthermore, carnosic acid reduces the expression levels of proinflammatory cytokines in human and mouse cells (Liu et al., 2019; Hosokawa et al., 2020) and regulates fatty acid metabolism (Park and Mun, 2013).

However, in ruminants, studies evaluating carnosic acid have been mainly correlated with an increase in meat quality through reduction of lipid oxidation (Nieto et al., 2010; Moran et al., 2012; Jordan et al., 2014). Surprisingly, its potential use as a strategy to reduce the oxidative and metabolic stress of transition dairy cows remains terra incognita. Based on the above, we hypothesized that providing carnosic acid during early lactation might minimize the typical exposure to oxidative stress and attenuate the typical inflammatory response during the peripartum period while allowing cows to reach peak performance. The objective of the present study was to evaluate the effects of a compound found in rosemary plants (*Salvia rosmarinus*) named carnosic acid during the transition period of dairy cows.

Materials and methods

Experimental design and treatments

The Institutional Animal Care and Use Committee (IACUC) of the South Dakota State University approved all the procedures for this study (protocol no. 2003-017A). The experiment was conducted from September to December 2020 at the South Dakota State University Dairy Research and Training Facility (Brookings, SD). Twenty late pregnant multiparous Holstein dairy cows were used in a randomized complete block design from −21 days prior to expected calving until 21 days in milk (DMI). Cows were blocked according to expected calving day, parity, and previous lactation milk yield, then assigned into one of two treatments. A total of 4 cows were removed from the experiment due to calving outside the range of −7 to 7 d relative to the expected calving date (n = 2), displaced abomasum (n = 1), and euthanasia due to low calcium/potassium at calving coupled with a lack of response to treatment (n = 1) (Table 1). Therefore, the number of experimental animals used for analysis was eight per treatment.

From day 1 to 3 after calving, cows received either a daily intravenous infusion of 500 mL of sterile saline solution (NaCl 0.9%; Saline; n = 8) or an infusion with carnosic acid at a rate of 0.3 mg/kg of BW (CA; n = 8). The complete dose of carnosic acid (Combi-Blocks, San Diego, USA, cat. number QC-4383) based on BW was supplied in a total volume of 500 mL of sterile saline solution. Treatments were infused into the external jugular vein before evening milking (1700 h).

In rodents, pharmacokinetic studies have been performed using intravenous infusions with CA at a rate of 10 or 20 mg/kg (Yan et al., 2009; Doolaege et al., 2011). To the authors' knowledge, a carnosic acid pharmacokinetic study in ruminants has never

been performed. Milk is the only body fluid that has been used to detect carnosic acid in ruminants supplemented with distilled rosemary leaves (Jordan et al., 2010). Jordan et al. (2010) fed goats a basal diet supplemented with 0, 10, and 20% of the diet with a pellet containing barley and distilled rosemary leaves and observed a carnosic acid concentration in milk ranging from 0.31 to 0.77 mg/kg. Therefore, given the limitations on pharmacokinetic data on carnosic acid in ruminants and the cost of procuring the CA coupled with the large amount of CA needed to supply the corresponding dose for an adult dairy cow, a 0.3 mg/kg BW dose was adopted as the most feasible daily dosage from 1 to 3 d postpartum.

Animal management

Cows were enrolled in the experiment from early September 2020 to early January 2021. Weather data from Mesonet at South Dakota State University (https://climate.sdstate.edu/) was used to evaluate the daily ambient temperature during the experimental period. Cows were fed using an individual gate system (American Calan, Northwood, NH, USA) and intakes were recorded daily. Diets were formulated using the Cornell Net Carbohydrate and Protein System (CNCPS) model contained within the Agricultural Modeling and Training Systems (AMTS) CattlePro diet-balancing software (version 4.16.1, AMTS LLC, Lansing, NY, USA) to meet the requirements of the average cow in the group (Table 2). Dry matter content of feed ingredients was determined once a week throughout the experiment, and diets were adjusted accordingly to maintain formulated DM ratios.

During the dry period, cows were housed in bedded pack pens. Immediately after calving, cows were reallocated in individual pens bedded with straw. On day 3, after

calving, cows were moved to a lactation free-stall barn. Cows were fed once daily (6:00) and milked twice daily (6:30 and 18:00). Body weight was measured weekly for each cow in the morning (9:00). Body condition score (BCS) (scale 1 = thin to 5 = obese) was assigned weekly by two individuals, and the average score was used for statistical analysis. All cows received the same close-up diet (1.46 Mcal of NE_L/kg and 15.1% CP; Table 2) and lactation basal diet (1.75 Mcal of NE_L/kg and 19.6% CP; Table 2), as a total mixed ration.

Blood collection and analyses

Blood was sampled from the coccygeal vein before morning feeding using a 20-gauge vacutainer needle (Becton Dickinson, Franklin Lakes, NJ) at –7, 2, 5, 7, 14, and 21 d relative to parturition. Blood was collected into evacuated tubes (BD Vacutainer, Becton Dickinson, Franklin Lakes, NJ) containing either serum clot activator or lithium heparin. After collection, tubes that contained lithium heparin were placed on ice, and tubes with serum clot activator were kept at 21 °C until centrifugation. Serum and plasma were obtained by centrifugation at 1,300 × g for 15 min at 21 °C and 4 °C, respectively. The aliquots were frozen at −80°C until further analysis.

Blood samples were analyzed for biomarkers related to energy metabolism [i.e., glucose, β-hydroxybutyric acid (BHB), non-esterified fatty acids (NEFA)], muscle mass catabolism (i.e., urea and creatinine), inflammation (i.e., ceruloplasmin and haptoglobin), liver function [i.e., albumin, bilirubin, glutamic-oxaloacetic transaminase (GOT), γ-glutamyltransferase (GGT), cholesterol, and paraoxonase (PON)], and oxidative stress [i.e., myeloperoxidase (MPO), reactive oxygen metabolites (ROM), ferric reducing antioxidant power (FRAP), nitrates (NO_3-), nitrites (NO_2-), nitric oxide metabolites

(NO_x), and oxygen radical absorbance capacity (ORAC)]. Furthermore, we calculated the ratios between oxidants and antioxidant defenses, e.g., ROM/FRAP and ROM/ORAC. Those ratios provide an integrated oxidant status index, which better assesses changes in oxidative status during the transition period when compared with the biomarkers alone (Abuelo et al., 2013; Mezzetti et al., 2019b).

Albumin, cholesterol, bilirubin, urea, creatinine, GOT, GGT, and glucose were analyzed using the IL Test purchased from Instrumentation Laboratory Spa (Werfen Co., Milan, Italy) in the ILAB 600 clinical auto-analyzer (Instrumentation Laboratory, Lexington, MA), following the procedures described previously (Trevisi et al., 2012; Batistel et al., 2016; Jacometo et al., 2016). Haptoglobin was analyzed using the method described by Skinner et al. (1991), while ceruloplasmin was determined based on Sunderman and Nomoto (1970) with modifications described by Jacometo et al. (2015). Antioxidant potential was assessed as ferric reducing antioxidant power (FRAP) using a colorimetric method (Benzie and Strain, 1996). Reactive oxygen metabolites (ROM) were analyzed with the d-ROMs-test (cod. MC002), purchased from Diacron (Grosseto, Italy). Paraoxonase, NO_x, NO_2-, and NO_3- were analyzed according to methods described by Trevisi et al. (2013). Myeloperoxidase was determined via colorimetry based on the reaction of MPO contained in the plasma sample with hydrogen peroxide, which forms H_2O and O−; the O− dianisidine dihydrochloride, and electron donor, reacts with the O−, releasing H_2O and a colored compound, which is then measured (Bionaz et al., 2007; Jacometo et al., 2015). Non-esterified fatty acids and BHB were measured using kits from Wako (Chemicals GmbH, Neuss, Germany) and Randox (Randox Laboratories Ltd., Crumlin, UK), respectively, following the procedures described previously (Bionaz

et al., 2007; Trevisi et al., 2012; Osorio et al., 2013). Finally, total antioxidants were assessed through the oxygen radical absorbance capacity (ORAC) assay. This method estimates the antioxidant capacity to inhibit phycoerythrin hydroxyl radical damage over time (Cao and Prior, 1999).

Milk and feed samples

Total mixed ration samples were collected weekly and frozen at −20 °C after DM analysis until further nutrient profile analysis. Monthly composites were analyzed for DM, CP, NDF, and ADF contents, while NEL was calculated using wet chemistry methods at a commercial laboratory (Dairy One; Ithaca, NY, USA).

Consecutive morning and evening milk samples were collected once weekly until 21 DMI. Composite milk samples were performed in proportion to milk yield at each milking, preserved (Broad Spectrum Microtabs II, Advanced Instruments, Norwood, MA), and analyzed for fat, protein, lactose, solids, milk urea nitrogen (MUN), and somatic cell count (SCC) (Dairy One; Ithaca, NY). Energy corrected milk (ECM) was calculated based on milk yield and milk sample analysis as follows: ECM = (12.82 × fat yield (kg)) + (7.13 × protein yield (kg)) + (0.323 × milk yield (kg)) (Hutjens, 2010).

Energy balance (EB) for each cow was calculated based on equations described previously (NRC, 2001). The net energy intake (NE_I) was determined based on daily DMI multiplied by NE_L density of the diet, and net energy of maintenance (NE_M) was calculated as $BW^{0.75} \times 0.080$. Requirements of net energy of lactation (NE_L) were calculated as NE_L = (0.0929 × fat % + 0.0547 × protein % + 0.0395 × lactose %) × milk yield. The net energy requirement for pregnancy (NE_P) was calculated as NE_P = [(0.00318 × day of gestation − 0.0352) × (calf birth weight/45)]/0.218. The equation used

to calculate prepartal EB (EB_{PRE}; Mcal/d) was $EB_{PRE} = NE_I - (NE_M + NE_P)$ and EB_{PRE} (as % of requirements) = $[NE_I / (NE_M + NE_P)] \times 100$. Finally, to calculate postpartal EB (EB_{POST}), the equation used was EB_{POST} (Mcal/d) = $NE_I - (NE_M + NE_L)$ and EB_{POST} (as % of requirements) = $[NE_I / (NE_M + NE_L)] \times 100$.

Phagocytosis capacity, oxidative burst activity, and L-selectin assay

The phagocytosis capacity and oxidative burst activity of peripheral leukocytes were determined upon challenge with enteropathogenic bacteria (*Escherichia coli* 0118:H8, kindly donated by M. A. Ballou, Texas Tech University, Lubbock) as previously described by Hulbert et al. (2011), with modifications. Briefly, blood was sampled on 21 d relative to calving from the coccygeal vein into evacuated tubes (BD Vacutainer, Becton Dickinson, Franklin Lakes, NJ) containing lithium heparin. Tubes were placed on ice and immediately transported to the laboratory for analysis.

Two hundred microliters of whole blood with 40 µL of 100 µM dihydrorhodamine 123 (Sigma-Aldrich, St. Louis, USA), and 40 µL of propidium iodine-labeled bacteria (109 CFU/mL) were incubated in a warm bath at 38.5°C for 10 min. Red blood cells were lysed through a sequence of washings with ice-cold deionized water and 5× PBS. After lysing, cells were resuspended in 1× PBS solution and stained for L-selectin, with BAQ92A primary anti-bovine CD62L antibody (Washington State University, Pullman, USA) and APC-labeled secondary antibody (Biolegend, San Diego, USA). Lastly, the cells were resuspended in 1× PBS solution for flow cytometry analyses (Attune™ NxT Flow Cytometer, Thermo Fisher Scientific, Waltham, USA). Data are reported as percentages of cell phagocytosis, oxidative burst, and L-selectin expression.

Statistical analysis

The effects of carnosic acid blood biomarkers were evaluated separately at 2 d relative to parturition (during infusions) and from 5 to 21 d postpartum as residual effects of CA. Performance data and residual effects were evaluated by repeated measures using the MIXED procedure of SAS 9.4 (SAS Institute Cary NC, USA). The statistical model contained the effects of treatment, time (day or week), and their interactions as fixed effects, while the cow within treatment was considered as a random effect. Single time-point data were analyzed following the same model, without the time statement. Blood biomarkers were log-scale transformed if needed to comply with normal distribution of residuals.

Residual data on blood biomarkers from 5 to 21 d postpartum was unequally spaced; therefore, the Spatial Power covariance structure was used for this analysis. For the equally spaced measures, the covariance structure was chosen between first-order autoregressive and heterogeneous first-order autoregressive based on goodness of fit (smaller Akaike information criteria). Covariates, including previous 305 d milk yield, prepartum DMI, ambient temperature, and blood metabolites at –7 d relative to calving, were maintained in the model when $p \leq 0.20$. Observations were considered outliers when Cook's distance > 0.50 and consequently excluded from the analysis. The CORR procedure of SAS was used to test the Pearson correlation coefficient (r) between milk performance and prepartum DMI, BW prepartum, and change in energy balance. The occurrence of health problems was analyzed using the FREQ procedure of SAS and interpreted based on Fisher's exact test probabilities. However, none of the health issues

observed in this experiment were affected ($p \geq 0.26$) by treatment (Table 1). Statistical significance was declared at $p \leq 0.05$ and tendencies at $p \leq 0.10$.

Results

Peripartal DMI, BW, and BCS

Main effects and interactions for prepartum and postpartum BW, BCS, DMI, DMI as % of BW, and EB are presented in Table 3. Results show a Trt × T interaction ($p \leq 0.05$) for prepartum DMI as % of BW and EB. However, no differences ($p > 0.10$) were observed between treatments at any given time point, and the interaction seems to be driven by changes in DMI as % of BW and EB from wk −2 to −1 observed for CA cows. Moreover, CA cows had lower ($p = 0.03$) postpartum DMI as % of BW, while a trend ($p = 0.06$) for greater BW prepartum was observed compared to saline cows. In contrast, treatment effects ($p > 0.10$) were not observed for postpartum BW. Body condition score and DMI were similar ($p > 0.10$) between treatment groups throughout the experimental period. Although EB was similar ($p > 0.10$) between treatments, further analysis showed that CA cows had a greater ($p = 0.04$) EB change ($EB_{prepartum} - EB_{postpartum}$) than saline cows around parturition (−19.8 vs. −13.8 ± 1.9 Mcal/d).

Milk production and composition

The main effects and interactions for postpartum production variables and milk composition are presented in Table 4. A trend ($p \leq 0.10$) for greater milk yield (Figure 1), ECM, and milk efficiency as milk/DMI was observed in CA cows in comparison with saline cows. The CA cows produced 4.5 kg/d and 6.3 kg/d more milk and ECM than saline cows, respectively. Similar to milk yield, a trend ($p = 0.10$) was observed for greater milk protein yield in CA cows when compared to the saline group. Milk yield was

negatively correlated with energy balance change around parturition (r=−0.63, p = 0.01) ($EB\ change = EB_{prepartum} - EB_{postpartum}$). In contrast, milk yield and BW prepartum were not correlated in either CA (r = −0.24; p = 0.56) or saline (r = 0.27; p = 0.52) cows (Figure 2).

Blood biomarkers

Immunometabolic effects of carnosic acid were evaluated at 2 d relative to calving and are present in Table 5. The CA cows had lower ($p \leq 0.05$) concentrations of haptoglobin, paraoxonase, FRAP, and NO_2^- than saline cows, while a trend (p = 0.07) for lower myeloperoxidase was observed in CA cows compared to saline cows.

Residual effects of treatments on blood biomarkers are shown in Table 6. There was a Trt × T interaction ($p \leq 0.05$) for haptoglobin, where CA cows had lower (p = 0.03) haptoglobin than saline cows at 5 d relative to parturition (Figure 3). A trend (p = 0.10) for lower NO_3^- in CA cows than saline was observed from 5 to 21 d relative to parturition (Table 6).

Phagocytosis capacity, oxidative burst activity, and L-selectin assays

Main effects for leukocyte phagocytosis capacity, oxidative burst activity, and L-selectin expression at 21 d relative to parturition are presented in Table 7. No differences ($p \geq 0.10$) were observed between treatments in the evaluated parameters.

Discussion

Performance parameters

Antioxidants are frequently part of dairy cows' diets and play a key role in minimizing harmful consequences of excessive production of reactive oxygen and nitrogen species, thereby improving their health status and reducing disease incidence

(Abuelo et al., 2015; Sordillo, 2016). Several studies have recently evaluated the effects of plant-based bioactive compounds with antioxidant properties as alternatives to conventionally available forms. In terms of animal productivity, performance responses when supplementing alternative plant-based antioxidants were not consistent among studies, most likely due to different properties of compounds, doses, animal species, among other reasons (Oh et al., 2017; Lopreiato et al., 2020). For example, Oh et al. (2015) observed that oxidative stress markers were not affected when supplementing lactating dairy cows with Capsicum oleoresin. However, the authors observed increased ECM and neutrophil activity. In contrast, Mezzetti et al. (2020) reported positive effects in terms of liver function and redox balance when transition dairy cows were supplemented with *Aloe arborescens*; however, milk production was unaffected by treatment.

In the present study, we observed that carnosic acid infusions increased milk yield, ECM, and milk protein yield. Moreover, there was no difference in DMI between treatment groups, which resulted in greater milk efficiency in terms of milk yield/DMI. Although the effects of carnosic acid in dairy cows remain to be elucidated, dietary supplementation of rosemary plant (*Salvia rosmarinus*) to dairy goats and sheep has been evaluated in terms of performance but with inconsistent results. For instance, studies had reported an increase in milk yield when small ruminants were fed a diet supplemented with dried rosemary plant or extract (Chiofalo et al., 2010; Smeti et al., 2015; Kholif et al., 2017), but this effect was not observed by others (Jordan et al., 2010). These benefits of dietary rosemary on lactation performance have been mainly associated with modifications in the ruminal environment by the interaction of compounds in rosemary

(e.g., carnosic acid) with rumen microbiota (Chiofalo et al., 2010; Smeti et al., 2015; Kholif et al., 2017). These studies suggest that the phenolic compounds found in rosemary leaves could inhibit protein degradation in the rumen, affect fatty acid metabolism during ruminal biohydrogenation, and enhance nutrient digestibility and ruminal fermentation (Smeti et al., 2015; Kholif et al., 2017). Therefore, a direct comparison between the effects of dietary rosemary plant or extract and intravenous infusion of carnosic acid is challenging. However, if further research confirms this effect, rosemary or rosemary compounds may influence milk yield in ruminants.

The lower DMI as % of BW aligned with greater EB change from pre- to postpartum observed in CA cows, suggesting a greater lipid mobilization. However, the difference in EB postpartum did not reach statistical significance (Table 3). In light of these results, we theorize that carnosic acid may influence liver lipid metabolism in transition dairy cows. In fact, carnosic acid reduces hepatic lipid accumulation in mice models through down-regulation of de novo lipogenesis and up-regulation of fatty acid oxidation signals at the mRNA and protein level, including PPARA (Park and Mun, 2013) and MAPK (Wang et al., 2012).

In transition dairy cows, increased fatty acid catabolism in the liver would induce transmission of signals towards the brain satiety center, reducing feed intake, as explained by the hepatic oxidation theory (Allen and Piantoni, 2013). Moreover, in this scenario, gluconeogenesis may be enhanced due to the higher availability of its precursors, e.g., pyruvate and oxalacetate. This theory could explain the lower DMI as % BW and the increase in milk production in CA cows. In fact, biomarkers for energy and liver function suggest that CA cows were able to cope with increased milk production

without increased liver damage and risk of health disorders such as ketosis, as evidenced by BHB concentration, which was similar between groups. However, we emphasize that the effects on hepatic metabolism here proposed are largely speculatory, and further studies are necessary to determine the specific mechanisms of how hepatocytes respond to carnosic acid under a peripartal condition in dairy cows.

Inflammation and liver function

Haptoglobin is a positive acute phase protein (APP) synthesized mainly by hepatocytes during an inflammatory process (Eckersall and Bell, 2010; Ceciliani et al., 2012). Higher haptoglobin concentrations are related to increased levels of proinflammatory cytokines, such as tumor necrosis factor alpha (TNFα), IL-1, and IL-6 (Bionaz et al., 2007). In dairy cows, haptoglobin levels are commonly increased during peripartum, with peak concentrations around the first week of lactation (Bionaz et al., 2007). This protein is a highly sensitive indicator of immune system activation and, as such, is a reliable biomarker of stress (Ceciliani et al., 2012; Bertoni and Trevisi, 2013). Additionally, haptoglobin is a major hemoglobin binding protein, limiting oxidative tissue damage mediated by hemoglobin. The induction of haptoglobin typically associated with enhanced oxidative stress seems to be a mechanism to prevent excessive damage caused by free radicals (Bertaggia et al., 2014).

In the present study, we observed a treatment effect on haptoglobin concentrations between the days of infusion (2 d) until 5 d relative to parturition (Table 5 and Figure 3, respectively). Several studies have observed the anti-inflammatory effects of carnosic acid (Oh et al., 2012; de Oliveira et al., 2018; Hosokawa et al., 2020). This phenolic diterpene significantly decreased the protein expression levels of various

proinflammatory cytokines in serum and tissues, e.g., TNFα, IL-1β, and IL-6 (Liu et al., 2019), through the suppression of the jun-N-terminal kinase (JNK), nuclear factor κB (NF-κB) and signal transducer and activator of transcription 3 (STAT3) pathways (Song et al., 2018; Hosokawa et al., 2020). Concentrations of haptoglobin in the days following calving observed in our study suggest a lower inflammatory status in CA cows, which agrees with previous studies using different animal models (Lopreiato et al., 2020).

Haptoglobin and PON are APP produced mainly by the liver. However, while haptoglobin is a positive APP, PON is characterized as a negative APP, meaning its hepatic synthesis is impaired during inflammation (Bionaz et al., 2007; Bertoni and Trevisi, 2013). In the plasma, PON is transported in association with high-density lipoproteins, and it exerts an important antioxidant function by hydrolyzing lipid hydroperoxides generated during oxidative stress (Turk et al., 2004; Turk et al., 2005). Moreover, decreased PON activity following parturition, especially in high producing dairy cows, is associated with intense lipomobilization and fat deposition in hepatocytes, related to liver damage or dysfunction (Turk et al., 2004).

In the present study, lower concentrations of PON were observed for CA cows than saline cows at 2 d relative to parturition (58 vs 67 U/mL). However, there was no difference between treatments from 5 to 21 d (83.1 vs 92.1 ± 5.3 U/mL). The reduction of PON activity at 2 d relative to parturition could be an indication of increased inflammation, intense lipomobilization, reduced levels of blood HDL, oxidative stress, or a combination of those (Bionaz et al., 2007). However, literature suggests that, regardless of treatment, PON activity observed throughout our study was higher than PON activity associated with impaired liver function or adverse health conditions after parturition

(Bionaz et al., 2007; Ceciliani et al., 2012). For example, Bionaz et al. (2007) evaluated the relationship between PON activity during the first month of lactation with health problems, inflammatory conditions, and liver function. Cows classified as lower quartile (43.8 ± 12.7 U/mL) based on PON activity presented lower milk yield, higher blood ROM, and greater occurrence of serious infections when compared with cows in the upper quartile (92.0 ± 19.8 U/mL).

In addition to blood biomarkers, immunological function and activation were evaluated through oxidative burst, phagocytosis, and L-selectin expression at 21 d relative to parturition. L-selectin is an important adhesion protein expressed on the surface of neutrophils and other leukocytes, being essential for the adhesion and rolling of immune cells across the endothelium (Kimura et al., 1999; Aleri et al., 2016). The expression of L-selectin on the surface of neutrophils decreased at calving, likely due to the immunosuppressive effects of glucocorticoids, but to recover within 1 to 3 d after parturition (Kimura et al., 1999; Sordillo, 2016; LeBlanc, 2020).

Furthermore, according to LeBlanc (2020), phagocytosis and oxidative burst are the two best-studied elements of neutrophil function, the first being the engulfing of invading microorganisms and the second an oxidative mechanism in which reactive oxygen metabolites are produced to destroy the ingested invading pathogen (Ingvartsen and Moyes, 2013). Along with decreased L-selectin expression, neutrophils exhibit impaired phagocytosis and oxidative burst activity around parturition (Sordillo and Streicher, 2002), which further contributes to the increased risk of infectious diseases during this period (Lopreiato et al., 2020). Our results showed no residual effects of CA on leukocyte function and activation at 21 d relative to calving. Regardless of treatment,

phagocytosis activity was lower and oxidative burst greater than reported by Zhou et al. (2016) and Batistel et al. (2018) around the third week of lactation. In agreement with the present results, Wu et al. (2017) also observed low percentages of neutrophil phagocytosis against *E. coli* (< 10%) from 28 d before to 28 d after calving. Moreover, our results regarding the other biomarkers for liver function support the idea that carnosic acid infusion from 1 to 3 d relative to parturition did not negatively affect the liver of the experimental animals.

Oxidative stress

Around 2 d relative to parturition, we also observed a treatment effect on plasma MPO, in which CA cows had lower concentrations than saline cows. MPO is a lysosomal peroxidase enzyme mainly stored in the azurophilic granules of neutrophils and to a lesser degree in primary lysosomes of monocytes (Depreester et al., 2017; Aratani, 2018). Upon immune cell activation, the enzyme is released extracellularly and into phagosomal compartments. In the presence of H_2O_2 and a halide (chloride, bromide, or thiocyanate), MPO catalyzes the formation of reactive oxygen intermediates, including hypochlorous acid (HOCl) (Rayner et al., 2014; Aratani, 2018). MPO is an important component of the host innate immune system against invading microorganisms. Thus it has been used as an inflammation biomarker (Depreester et al., 2017).

Reactive oxygen species produced by MPO have been implicated as mediators of host oxidative tissue damage and cellular dysfunction (Depreester et al., 2017; Aratani, 2018). Our results agree with previous studies, which observed an effect of carnosic acid on decreasing the activity of MPO in mice immune cells during acute lung injury (Li et al., 2019) and acute colitis (Yang et al., 2017). According to these studies, carnosic acid

may attenuate oxidative stress by suppressing the production of proinflammatory cytokines, immune cells activation, and migration. Lower levels of MPO aligned with haptoglobin results, suggesting lower inflammation in CA cows than saline cows around the days of CA infusion.

In contrast with our initial hypothesis, blood ROM was not affected by treatment. These results contrast with previous *in vitro* studies that observed a lower accumulation of ROS when different cell lines were treated with carnosic acid (Liu and Dong, 2017; Peng et al., 2020; Lee and Jang, 2021). de Oliveira et al. (2015) found lower ROS concentrations when neuroblastoma cells were treated with carnosic acid during induced neurotoxicity. However, the authors observed no differences when cells were not challenged. Overall, literature suggests that the effect of carnosic acid on the production of reactive oxygen compounds might be dose-dependent, and cellular conditions may have an important role in its effectiveness. Alternatively, the lack of effect on ROM due to CA infusion could be attributed to CA attenuating ROS production at a phagocytic cell level, but not sufficient to affect ROS being produced during the high metabolic state at the onset of lactation. Moreover, the limitation of *in vivo* experiments, especially in ruminants, makes it particularly challenging to correlate our findings with a specific biological mechanism.

The high complexity of the oxidant defense system and dynamics between prooxidants and antioxidants among different tissues makes it challenging to quantify and infer the overall whole-organism redox balance accurately. The individual quantification of specific antioxidants does not provide a complete picture of the antioxidant capacity in a given sample since multiple mechanisms act synergically to counterbalance oxidative

stress (Benzie and Strain, 1996; Abuelo et al., 2015). In this context, different analytic methods, i.e., FRAP and ORAC, were developed to estimate antioxidant activity of a certain sample. An increase in FRAP plasma values indicates a greater need for neutralizing ROM production, while higher ORAC denotes greater protection produced by antioxidants in plasma (Batistel et al., 2016).

The CA cows had a lower FRAP at 2 d relative to parturition when compared to saline cows. However, no differences were observed for ORAC, ROM/FRAP, or ROM/ORAC. Plasma concentrations of carnosic acid declined rapidly after CA intravenous infusion in rats, while gastrointestinal absorption results in longer plasma CA retention (Yan et al., 2009; Doolaege et al., 2011). In the present study, blood samples were collected around 12 h after intravenous infusions, which might help explain why plasma antioxidant activity and oxidative status were mostly unaffected by carnosic treatment. Furthermore, studies with carnosic acid in ruminant species are scarce, and the effects of its supplementation on plasma FRAP or ORAC are currently unknown. Jordan et al. (2014) evaluated the level of transfer of two typified rosemary extracts (carnosic acid and carnosol) in lamb tissues after dietary supplementation. Carnosic acid, especially at 1:1 ratio with carnosol, increased muscle and liver FRAP, although plasma FRAP was not evaluated in that study.

Carnosic acid treatment reduced levels of NO_2- around the period of infusions (2 d postpartum). Moreover, a residual effect of treatment was observed in terms of NO_3- from 5 to 21 d relative to parturition. Nitrite (NO_2^-) and nitrate (NO_3^-) are stable products of nitric oxide (NO), and the measurement of these oxidation products is used to estimate NO production in biological fluids (Levine et al., 1998; Batistel et al., 2016).

Nitric oxide is an important free radical synthesized by immune and endothelial cells; it is involved in many biological functions, including intracellular communication, vasodilatation, and inflammation (Burner et al., 2000; Tripathi et al., 2007; Nash et al., 2012). At physiological levels, its toxicity is generally limited (Nash et al., 2012). However, at high concentrations, NO is rapidly oxidized to reactive nitrogen oxide species (RNOS), e.g., nitrogen dioxide (NO_2) and dinitrogen trioxide (N_2O_3), which in turn induce cell toxicity through modification of enzymes, signaling proteins, and transcription factors (Coleman, 2001). Studies have observed decreased levels of NO in the presence of carnosic acid, which was associated with lower levels of the inducible NO synthase enzyme (iNOS) (Hou et al., 2012; Oh et al., 2012). Overall, our results indicate that carnosic acid may have decreased NO synthesis, suggesting lower inflammation and a lower risk of oxidative damage.

Conclusions

The findings of this study revealed that carnosic acid reduced inflammation and oxidative stress biomarkers around the period of CA infusion. In addition, carnosic acid contributed to an increase in lactation performance, although the mechanisms responsible for this response remain unclear. Pharmacokinetics studies are necessary to further understand how carnosic acid is metabolized in ruminants and how fast metabolism occurs to estimate optimal dosage and carnosic acid supplementation through its plant-based source, rosemary. Overall, the results presented here describe the potential benefits of providing antioxidants such as carnosic acid during the peripartal period of dairy cows.

Table 2.1. Frequency of occurrence of health problems in peripartum dairy cows receiving an intravenous infusion of carnosic acid (CA) or saline (Saline) from -21 relative to parturition through 21 DIM.

	Treatment	
Event	**CA**	**Saline**
Total animals	10	10
Subclinical ketosis[1]	5	4
Clinical ketosis[2]	0	2
Retained placenta[3]	0	1
Displaced abomasum	0	1
Metritis[4]	1	0
Pneumonia	1	0
Total excluded cows[5]	2	2

[1] Defined as cows having 1.4 to 2.9 mmol/L ketone concentration in blood, detected using a Precision Xtra analyzer (Abbott Labs). Treatment administrated was oral propylene glycol and injection of vitamin B complex.
[2] Defined as cows having > 2.9 mmol/L ketone concentration in blood, detected using a Precision Xtra analyzer (Abbott Labs). Treatment administrated was oral propylene glycol and injection of vitamin B complex.
[3] Defined as fetal membranes retained > 24 h postpartum.
[4] Foul-smelling, watery-consistency uterine discharge after calving.
[5] Cows excluded from the experiment due to calving outside the range of −7 to 7 d relative to the expected calving date (CA, n = 1; Saline, n = 1), displaced abomasum (Saline, DIM = 18, n = 1), euthanasia due to low calcium/potassium at calving coupled with a lack of response to treatment (CA, DIM = 0, n = 1).

Table 2.2. Ingredient composition of diets during the close-up (−21 d to expected calving) and early lactation periods (21 d relative to calving).

Component	Diet	
	Close-up	**Lactation**
Ingredient, % of DM		
Corn silage	36.9	32.5
Alfalfa hay	-	7.6
Alfalfa haylage	-	12.1
Grass hay	24.6	-
Wheat straw	12.4	-
Soybean meal	11.5	5.7
Cottonseed	-	6.7
Molasses	-	5.0
Dry cow grain mix[1]	14.6	-
Lactating cow grain mix[2]	-	30.4
Chemical analysis		
DM, %	46.7	47.6
NEL, Mcal/ kg DM	1.46	1.75
CP, % DM	15.1	19.6
NDF, % DM	43.7	31.8
ADF, % DM	27.4	17.7
Starch, % DM	15.9	28.2
DCAD, mEq/100 g	−12.3	-

[1] Dry cow grain mix contained (as % DM): distillers grain dry (24.8), soy hulls (21.4), biochlor (18.5), limestone Ca (14.0), magnesium sulfate 7H2O (3.5), calcium phosphate 21% (3.5), reashure choline (2.8), calcium chloride dihydrate (2.3), magnesium oxide 54% (2.3), calcium sulfate dihydrate (1.5), chromium propionate 0.04% (1.0), DV nutritek (0.9), JPW dairy vitamin premix 8298.V05 (0.9), salt white (0.7), vitamin E 20000 IU/lb (0.7), JPW dairy TM premix 8298.E04 (0.7), DTX binder (0.4), rumensin 90 g/lb (0.1).

[2] Lactating cow grain mix contained (as % DM): corn grain ground fine (66.1), soy best (14.8), distillers grain dry (7.4), limestone Ca (2.7), sodium bicarbonate (2.8), energy booster 100 (2.2), salt white (1.16), urea (0.90), magnesium oxide 54% (0.55), calcium phosphate 21% (0.33), JPW dairy vitamin premix 8298.V05 (0.29), JPW dairy TM premix 8298.E04 (0.29), DV nutritek (0.22), vitamin E 20000 IU/lb (0.11), DTX binder (0.11), rumensin 90 g/lb (0.02), biotin 1% (0.02).

Table 2.3. Differences between treatment groups during prepartum and main residual effects of intravenous infusion of carnosic acid (CA) or saline solution (Saline) on BW, BCS, DMI, DMI as % of BW, and energy balance.

Parameter	Treatment		SEM[1]	*p*-Value		
	CA	Saline		Trt	Time	Trt × T[2]
Prepartum						
Body weight, kg	873.3	801.6	25.0	0.06	0.01	0.34
Body condition score	3.70	3.75	0.08	0.65	0.74	0.24
Dry matter intake, kg/d	15.3	13.2	0.92	0.12	<0.01	0.41
Dry matter intake, % BW	1.73	1.69	0.13	0.82	<0.01	0.04
Energy balance, Mcal/d	6.10	4.49	1.56	0.47	<0.01	0.05
Postpartum						
Body weight, kg	735.5	739.9	7.64	0.71	<0.01	0.19
Body condition score	3.55	3.54	0.04	0.88	<0.01	0.13
BW change[3], %	−13.0	−11.6	0.92	0.33	<0.01	0.22
BCS change[3], %	−4.8	−5.2	1.06	0.77	<0.01	0.26
Dry matter intake, kg/d	18.6	20.2	0.72	0.15	<0.01	0.70
Dry matter intake, % BW	2.48	2.85	0.11	0.03	<0.01	0.33
Energy balance, Mcal/d	−13.51	−9.53	1.82	0.14	0.02	0.19

[1] Largest standard error of the mean.
[2] Interaction of treatment and time (day or week) relative to calving.
[3] Percent change from the average of 3 weeks prepartum.

Table 2.4. Main residual effects of intravenous infusion of carnosic acid (CA) or saline solution (Saline) on milk production and composition parameters during 21 d in milk.

Parameter	Treatment		SEM[1]	*p*-Value		
	CA	Saline		Trt	Time	Trt × T[2]
Milk yield, kg/d	41.8	37.3	1.76	0.10	<0.01	0.35
Energy corrected milk[3], kg/d	51.3	45.0	2.35	0.08	<0.01	0.95
Milk efficiency, Milk/DMI[4]	2.37	2.04	0.12	0.06	0.02	0.56
Milk efficiency, ECM/DMI	2.75	2.47	0.17	0.25	0.03	0.11
Milk Composition						
Fat, %	5.07	5.26	0.23	0.56	<0.01	0.78
Fat yield, kg/d	2.11	1.89	0.12	0.19	0.13	0.82
Protein, %	3.52	3.53	0.14	0.96	<0.01	0.60
Protein yield, kg/d	1.45	1.27	0.07	0.10	0.18	0.93
Lactose, %	4.69	4.74	0.05	0.49	<0.01	0.56
Solids, %	14.35	14.62	0.25	0.47	<0.01	0.70
Milk urea nitrogen, mg/dL	12.71	12.19	0.92	0.70	0.73	0.88
Log-transformed SCC[5]	4.93	5.11	0.14	0.40	0.08	0.79

[1] Largest standard error of the mean.
[2] Interaction of treatment and time (day or week) relative to calving.
[3] Energy corrected milk (ECM), calculated as [12.82 × fat yield (kg)] + [7.13 × protein yield (kg)] + [0.323 × milk yield (kg)]; [4] DMI: dry matter intake; [5] SCC: somatic cell count.

Table 2.5. Effects of intravenous infusion of carnosic acid (CA) or saline solution (Saline) on blood biomarkers related to energy metabolism, inflammation, liver function, muscle body mass, metabolism, and oxidative stress in dairy cows at 2 d relative to parturition.

	Treatment			*p*-Value
Parameter	**CA**	**Saline**	**SEM[1]**	**Trt**
Energy metabolites				
Glucose, mmol/L	4.10	4.03	0.11	0.68
BHB, mmol/L	0.83	0.82	0.12	0.98
NEFA, mmol/L	0.83	0.65	0.13	0.35
Inflammation				
Ceruloplasmin, µmol/L	2.30	2.41	0.07	0.38
Haptoglobin[2], g/L	0.67	1.06	0.16	<0.01
Liver function				
Albumin, g/L	33.46	33.97	0.49	0.48
Cholesterol, mmol/L	1.74	1.84	0.09	0.42
Paraoxonase, U/mL	58.03	67.38	2.61	0.03
Bilirubin[2], µmol/L	5.17	5.21	0.42	0.99
AST, U/L	119.9	105.3	8.20	0.23
GGT[2], U/L	17.39	16.34	0.09	0.48
Muscle mass catabolism				
Urea, mmol/L	5.39	5.28	0.27	0.80
Creatinine, µmol/L	94.25	88.27	2.97	0.21
Oxidative stress				
FRAP [2] , µmol/L	140.1	163.1	0.06	0.02
Myeloperoxidase, U/L	485.3	519.6	12.8	0.07
ROM, mg H_2O_2/100mL	15.88	16.73	0.52	0.28
NO_2^-, µmol/L	2.63	4.58	0.35	<0.01
NO_3^-, µmol/L	21.85	19.62	1.13	0.19
NO_x, µmol/L	23.9	23.65	0.87	0.84
ORAC, µg/mL	12.50	13.06	0.34	0.25
ROM/FRAP	0.11	0.10	0.01	0.44
ROM/ORAC	1.25	1.32	0.06	0.40

[1] Largest standard error of the mean.
[2] Data were log-transformed before statistics. The standard errors of the means associated with log-transformed data are in log scale.

Table 2.6. Residual effects of intravenous infusion of carnosic acid (CA) or saline solution (Saline) on blood biomarkers related to energy metabolism, inflammation, liver function, muscle body mass, metabolism, and oxidative stress in dairy cows from 5 to 21 d.

	Treatment			*p*-Value		
Parameter	**CA**	**Saline**	**SEM[1]**	**Trt**	**Time**	**Trt × T[3]**
Energy metabolites						
Glucose, mmol/L	3.87	4.02	0.08	0.20	0.03	0.88
BHB, mmol/L	1.06	0.99	0.21	0.83	0.73	0.90
NEFA, mmol/L	0.63	0.63	0.09	0.99	<0.01	0.88
Inflammation						
Ceruloplasmin, µmol/L	2.88	2.99	0.12	0.55	0.28	0.90
Haptoglobin[2], g/L	0.44	0.44	0.17	0.99	0.14	0.04
Liver function						
Albumin, g/L	35.51	34.71	0.33	0.11	0.03	0.53
Cholesterol, mmol/L	2.92	3.11	0.13	0.31	<0.01	0.37
Paraoxonase, U/mL	83.08	92.07	5.33	0.25	0.10	0.79
Bilirubin[2], µmol/L	3.46	3.66	0.20	0.78	<0.01	0.79
AST, U/L	149.2	122.9	13.43	0.19	0.21	0.34
GGT[2], U/L	23.92	20.25	0.16	0.32	0.06	0.40
Muscle mass catabolism						
Urea, mmol/L	5.01	5.06	0.23	0.87	0.37	0.51
Creatinine, µmol/L	84.83	82.35	1.42	0.25	<0.01	0.71
Oxidative stress						
FRAP [2] , µmol/L	128.0	132.5	0.09	0.71	<0.01	0.12
Myeloperoxidase, U/L	447.4	436.1	19.9	0.70	0.01	0.95
ROM, mg H_2O_2/100mL	17.61	18.40	0.69	0.44	0.10	0.35
NO_2^-, µmol/L	5.78	5.90	0.42	0.85	<0.01	0.92
NO_3^-, µmol/L	21.71	23.07	0.58	0.10	0.01	0.91
NO_x, µmol/L	28.25	28.21	0.72	0.97	<0.01	0.96
ORAC, µg/mL	12.56	12.23	0.58	0.70	0.95	0.97
ROM/FRAP	0.14	0.15	0.01	0.86	<0.01	0.69
ROM/ORAC	1.48	1.56	0.09	0.53	0.74	0.56

[1] Largest standard error of the mean.

[2] Data were log-transformed before statistics. The standard errors of the means associated with log-transformed data are in log scale.

[3] Interaction of treatment and days relative to calving.

Table 2.7. Residual effects of intravenous infusion of carnosic acid (CA) or saline solution (Saline) on leukocyte phagocytosis capacity, oxidative burst activity, and L-selectin at 21 d relative to parturition.

	Treatment			*p*-Value
Parameter	**CA**	**Saline**	**SEM[1]**	**Trt**
Phagocytosis, %	9.34	8.97	1.16	0.82
L-selectin, %	67.0	71.7	6.55	0.63
Oxidative burst, %	88.0	85.3	2.33	0.41

[1] Largest standard error of the mean.

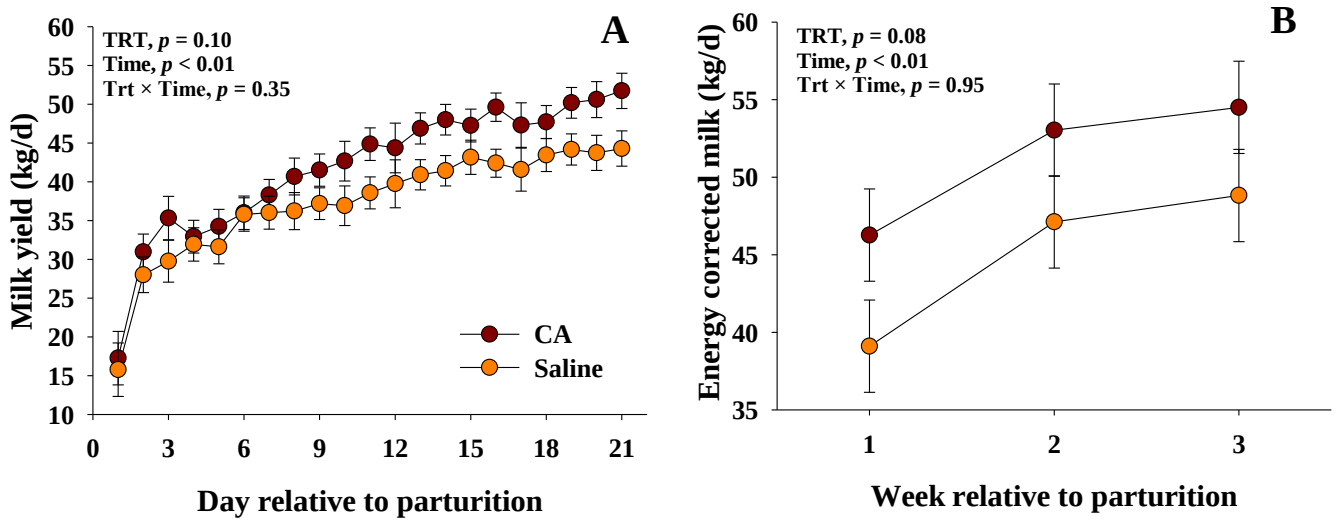

Figure 2.1. Milk yield (A) and energy corrected milk (B) for peripartal dairy cows infused from 1 to 3 d postpartum with 500 mL of sterile saline solution (Saline) or carnosic acid (CA) at a rate of 0.3 mg/kg BW supplied in a total volume of 500 mL of saline. Values are means, and the standard errors are represented by vertical bars.

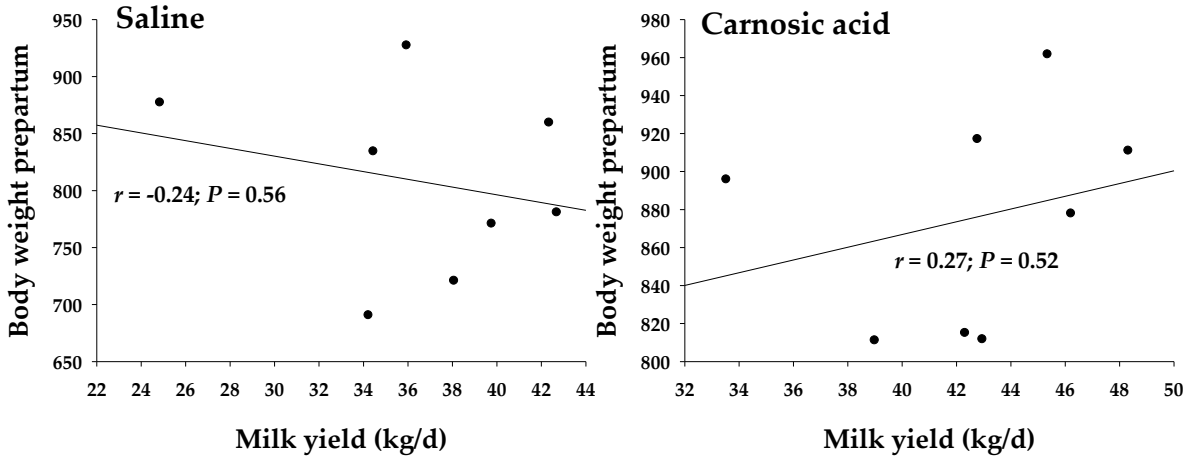

Figure 2.2. Correlation between body weight prepartum and milk yield for peripartal dairy cows infused with 500 mL of sterile saline or carnosic acid at a rate of 0.3 mg/kg BW supplied in a total volume of 500 mL of sterile saline solution.

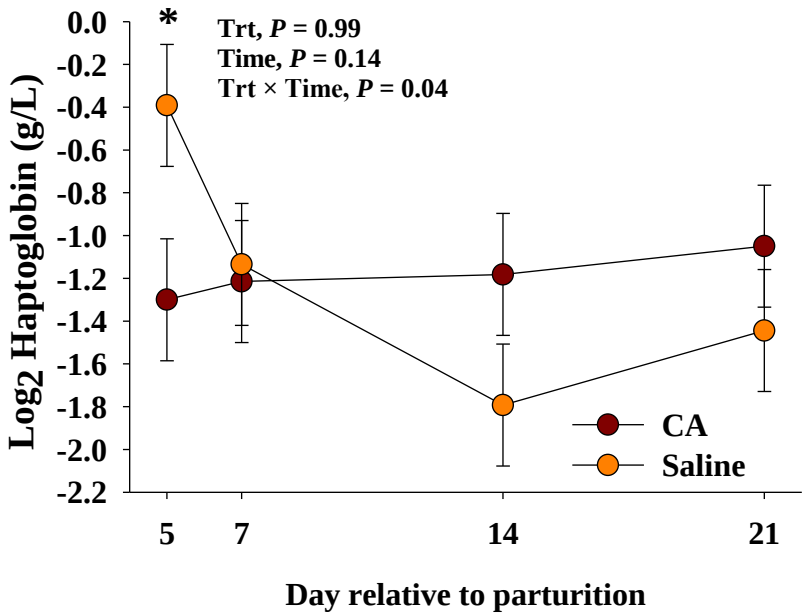

Figure 2.3. Residual effects of carnosic acid (CA) or saline intravenous infusions on blood biomarkers from 5 to 21 d relative to parturition for haptoglobin. Mean separations between treatments at a given time point were evaluated at a treatment × time interaction ($p \leq 0.10$), and differences (*) were declared at $p \leq 0.05$. Values are means and the standard errors are represented by vertical bars.

CHAPTER 3. EFFECTS OF CARNOSIC ACID ON HEPATOCYTES SUBJECTED TO HYDROGEN PEROXIDE-INDUCED OXIDATIVE CELL DAMAGE *IN VITRO*

Abstract

The objectives of this study were to investigate the oxidative damaging effects of hydrogen peroxide (H_2O_2) on alpha mouse liver 12 (AML12) cell line *in vitro*, determine whether CA has a cytoprotective effect on AML12 during induced oxidative stress, and study the underlying mechanisms by which CA can affect the liver in a mouse model. The AML12 cells were cultured in medium containing three different concentrations of CA for 12 h, followed by another 12 h incubation with H_2O_2, which was used to stimulate oxidative stress *in vitro*. Specific treatments were (i) control (culture medium without any treatment), (ii) H_2O_2 (500 µM), (iii) carnosic acid 3.0 mg/L + 500 µM H_2O_2, (iv) CA 6.0 mg/L + 500 µM H_2O_2, and (v) CA 9.0 mg/L + 500 µM H_2O_2. AML12 viability, production of ROS, and the activity of superoxide dismutase (SOD) and glutathione peroxidase (GPx) were measured. We observed a trend of a treatment effect on viability. In comparison with control, hydrogen peroxide reduced cells viability in all the treatments, except for CA 9.0 mg/L + 500 µM H_2O_2, which did not differ from control. Contrary to our initial hypothesis, CA treatment did not affect intracellular ROS production. Although no effect of treatments was observed on glutathione peroxidase activity, superoxide dismutase activity was positively affected by treatment. Moreover, CA increased superoxide dismutase activity linearly, indicating a dose-dependent response to CA. Results from this study indicate that carnosic acid supplementation

might be beneficial in periods of redox balance alterations *in vivo*, although further studies are necessary to determine dose-dependent responses and safety levels.

Keywords: AML12; oxidative stress; carnosic acid

Introduction

Oxidative stress is a metabolic condition that can be defined as the damage occurring to cellular macromolecules as a consequence of a serious imbalance between oxidants and antioxidants molecules (Halliwell, 2007a; Mavangira and Sordillo, 2018). Oxidative damage has negative effects, especially on lipids, proteins, and DNA, and has been associated with the development of several pathologies in various animal species, including dairy cows (Sordillo and Aitken, 2009; Turk, 2017) and humans (Taniyama and Griendling, 2003; Pizzino et al., 2017).

In dairy cows, oxidative stress is among the most critical challenges faced around parturition (Ma et al., 2019). During this period, cows undergo a shift from a gestational nonlactating state to a state of copious milk synthesis and secretion, demanding metabolic adjustments to address the dramatic increase in energy requirements (Sordillo and Raphael, 2013), which leads to an increase in oxygen requirements and the consequent production of oxidants (Sordillo and Aitken, 2009; Abuelo et al., 2015; Abuelo et al., 2016a). In parallel, the proinflammatory state of the transition cow leads to the release of proinflammatory cytokines, which can further induce ROS production by both phagocytic and non-phagocytic cells (Abuelo et al., 2015), increasing the alteration in redox balance.

One of the most important aspects of oxidative stress in transition dairy cows is negative energy balance (NEB) and the following increase in blood concentration of non-esterified fatty acids (NEFA). Blood NEFA starts to rise 2 to 3 wk before calving, peaks around parturition or at the first week post-partum (Ingvartsen and Andersen, 2000), and as NEFA increases, so does their uptake by the hepatic tissue (Busato and Bionaz, 2020).

When used as an energy substrate, NEFA enhance ROS production during β-oxidation (Schonfeld and Wojtczak, 2008; Abuelo et al., 2015). The subsequent alteration in redox balance may lead to insulin resistance in peripheral tissues, which further increases lipolysis and NEFA levels (Sordillo and Raphael, 2013; Hurrle and Hsu, 2017; Youssef and El-Ashker, 2017). In fact, the exposure of hepatocytes in culture to pathologically increased fatty acids levels have been shown to induce mitochondrial dysfunction and increase cellular ROS content (Gao et al., 2018). According to Schonfeld and Wojtczak (2008), within the cell, ROS production from fatty acids occurs mostly in the mitochondria through the respiratory chain, and in the plasma membrane, through NADPH oxidase.

During periods of high antioxidant demands such as the peripartal period, dietary supplementation with antioxidants is necessary to meet increased requirements (NRC, 2001; Spears and Weiss, 2008; Abuelo et al., 2019). Vitamin E and selenium are the most widely used antioxidants included in dairy cattle diets (Abuelo et al., 2015); however, in the last few decades, there has been an increased interest in studying alternative natural compounds with antioxidant and anti-inflammatory properties as supplements for food-producing animals (Yang et al., 2015; Ciampi et al., 2020; Lopreiato et al., 2020). Among

those alternative sources of antioxidants are polyphenol compounds, which encompass a family of 500 different phytochemicals and present potential therapeutic applications due to their intrinsic antioxidative and anti-inflammatory properties (Ballou et al., 2019; Lopreiato et al., 2020). The polyphenol compound carnosic acid (CA) is a phenolic diterpene found in herbs from the Labiatae family, especially common salvia (*Salvia officinalis*) and rosemary (*Salvia rosmarinus*) and has antioxidant and antimicrobial properties (Birtic et al., 2015; Loussouarn et al., 2017).

Carnosic acid has been demonstrated to play a role in oxidative stress response by the activation of the PI3K/Akt/NRF2 signaling pathway in human cells (de Oliveira et al., 2015; de Oliveira et al., 2016) and to reduce inflammation in human and mouse cells through the NF-κB signaling pathway (Liu et al., 2019; Hosokawa et al., 2020). Costa et al. (2007) observed a dose-dependent protective effect of CA (10, 20, and 30 μM) on Hep G2 cells exposed to aflatoxin B1. Furthermore, CA at 1 μM protects against oxidative stress-induced cytotoxicity in primary hepatocytes and AML12 cells (Wang and Takikawa, 2016). In contrast, Dickmann et al. (2012) observed a dose-dependent increase in hepatotoxicity caused by CA (0.5 to 2.5 μM) in three human hepatocyte donors, but without a concurrent increase in the apoptosis markers caspase-3/7.

Using mouse liver as a biological model, we hypothesized that CA would have a protective effect on cultured cells subjected to hydrogen peroxide-induced oxidative damage. The objectives of this study were to (a) investigate the oxidative damaging effects of hydrogen peroxide (H_2O_2) on the alpha mouse liver 12 (AML12) cell line *in vitro*, (b) determine whether CA has a cytoprotective effect on AML12 during induced

oxidative stress, and (3) study the underlying mechanisms by which CA can affect the liver in a mouse model.

Materials and methods

Cell culture and treatments

The AML12 (alpha mouse liver 12) cell line was purchased from the American Type Culture Collection (ATCC, Manassas, Virginia, USA) and maintained in DMEM:F12 base medium (catalog number 11320033, Gibco, Amarillo, Texas, USA) supplemented with 10% fetal bovine serum, 20 µg/mL of insulin, 5.5 ug/mL transferrin, 5 ng/mL selenium, and 40 ng/mL dexamethasone at 37°C and 5% CO_2 in a humidified incubator. Cells were revived from liquid nitrogen and after thawing, AML12 were cultured in 75cm² cell culture flasks. Cells were detached with trypsin 0.05% at 70 to 80% of confluence to perform the assays. Trypan blue exclusion was used to evaluate cell death. Cells were collected using trypsin, diluted 1:1 in trypan blue, and counted on a hemocytometer (Countess® II FL Automated Cell Counter, Life Technologies, Carlsbad, California, USA) to obtain the number of viable cells.

Carnosic acid (95% purity) was purchased at Combi-Blocks (San Diego, USA, catalog number QC-4383). Carnosic acid was primarily diluted to 0.5 mg/mL with molecular ethanol, then successively diluted at the time of challenge to reach the concentrations of 3, 6, and 9 mg/L with cell culture medium. The tested concentration of carnosic acid in the present study was chosen on the basis of previous results of Jordan et al. (2010). Those authors observed a carnosic acid concentration of 6.17 ± 0.62 mg/L in

plasma of sucking goat kids in which the dams were fed a basal diet with a 10% inclusion rate of distilled aromatic rosemary leaves.

For the cell treatment, indicated concentrations of carnosic acid were applied to the cells for 12 h. Following pretreatments, to establish *in vitro* oxidative stress, cells were incubated with H_2O_2 as previously described (Jin et al., 2016; Ma et al., 2019). Briefly, 30% H_2O_2 was diluted to 1 M stock using sterilized 1×PBS (100 μL 30% H_2O_2 was diluted with 870.3 μL 1×PBS). Hydrogen peroxide at 1M was further diluted with cell culture medium to 500 µM and then added into culture plates and incubated for 12 h. Hydrogen peroxide solutions were made fresh before use. The tested concentration and time of incubation of H_2O_2 in the present study were chosen based on previous studies (Jin et al., 2016; Ma et al., 2018; Ma et al., 2019). Specific treatments were (i) control (culture medium without any treatment), (ii) H_2O_2 (500 µM), (iii) carnosic acid 3.0 mg/L + 500 µM H_2O_2, (iv) CA 6.0 mg/L + 500 µM H_2O_2, and (v) CA 9.0 mg/L + 500 µM H_2O_2. Cell viability, production of ROS, and the activity of superoxide dismutase (SOD) and glutathione peroxidase (GPx) were measured.

Cell viability

Cells were plated at a concentration of 1 x 10^5 cells/mL in a 96-well plate and allowed to culture for 24 h before starting the experiment. Carnosic treatments and hydrogen peroxide-induced oxidative cell damage were applied as described above. The viability of AML12 was measured using the CellTiter-Glo 2.0 Cell Viability Assay (Promega Corporation Madison, Wisconsin, USA).

The method is based on cell lysis followed by generation and quantification of a luminescent signal proportional to the amount of ATP present, which indicates the presence of metabolically active cells. The assay has as a principle the mono-oxygenation of luciferin, catalyzed by luciferase in the presence of Mg^{2+}, molecular oxygen, and ATP, which is contributed by viable cells. Briefly, 96-well plates and reagents were cooled to room temperature before beginning the assay. The medium was removed from wells, and cells were washed with 1×PBS. Following washing, 100 µL of Opti-MEM media (catalog number 11058021, Gibco, Amarillo, Texas, USA) and 100 µL of CellTiter-Glo 2.0 reagent were added to each well. To induce cell lysis, the 96-well plate was mixed at 200 rpm for 5 min using an incubator shaker (BenchTop Lab Systems, Saint Lois, MO, USA). The plate was incubated at room temperature for an additional 25 min to stabilize the luminescent signal. Luminescence was measured using a Synergy 2 Multi-Detection Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA).

Intracellular ROS production

Cells were plated at a concentration of 1 x 10^5 cells/mL in a 96-well plate and allowed to culture for 24 h before starting the experiment. Treatments were applied as described above. The intracellular ROS production was measured with Reactive Oxygen Species (ROS) Detection Assay Kit (BioVision Inc., Milpitas, CA, USA). The assay is based on the presence of a cell-permeable fluorogenic probe. The oxidation of this fluorogenic probe by intracellular ROS yields a highly fluorescent product. The fluorescence intensity is proportional to the ROS levels.

Briefly, following treatments and hydrogen peroxide-induced oxidative cell damage, media was removed, and adherent cells were washed with 100 µL of ROS Assay Buffer. Then, 100 µl of 1X× ROS label diluted in ROS Assay Buffer were added to each well. Cells were incubated for 45 min in the dark at 37°C. Following incubation, ROS label was removed, and 100 µl of 1×PBS was added to each well. Fluorescence was measured immediately at Ex/Em= 495/529 nm using a Synergy 2 Multi-Detection Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA). Change in fluorescence was determined after background subtraction.

Detection of antioxidant enzymes

The activity of glutathione peroxidase (GPx) was determined using a colorimetric diagnostic assay kit purchased from BioVision Inc. (Milpitas, CA, USA), according to the manufacturer's protocols. Superoxide dismutase (SOD) was assessed using a colorimetric activity kit from Invitrogen (Invitrogen, San Diego, CA, USA). Briefly, cells were plated at a concentration of 2.5 x 10^5 cells/mL in a 6-well plate and allowed to culture for 24 h before starting the experiment. Treatments were applied as described previously. To prepare a cell pellet, the medium was removed, and 6-well plates were rinsed with 1×PBS. Cells were harvested by gentle trypsinization, and 1 x 10^6 cells were transferred to a microcentrifuge tube on ice. Microcentrifuge tubes were centrifuged at 250 × *g* for 10 min at 4°C, and the supernatant was discarded. The cell pellet was resuspended in ice-cold 1×PBS and centrifuged at 250 x g for 10 min at 4°C. The supernatant was discarded.

For the SOD assay, 0.5 mL of 1xPBS were added to the cell pellet, and the solution was transferred to a 2-mL RNase-free O-ring tube, containing one stainless steel bead, 5 mm (Qiagen, Hilden, Germany) and homogenized in a Beadbeater (BioSpec Products, Bartlesville, OK, USA) for 30s. After homogenization, the lysate was transferred to a 2-mL microtube and centrifuged at 1,500 × g for 10 min at 4°C. The supernatant was collected, and cell lysate was diluted at 1:3 with Assay Buffer. Samples were stored at -80◦C until analysis. The assay was performed as described by the manufacturer, with SOD standards and diluted samples plated in triplicate in a 96-well plate. Absorbance was read at 450 nm using a spectrophotometer (Varian Cary 50 UV-vis).

For the GPx assay, 0.2 mL of assay buffer were added to the cell pellet and this solution was transferred to a 2-mL RNase-free O-ring tube, containing one stainless steel bead, 5 mm (Qiagen, Hilden, Germany) and homogenized in a Beadbeater (BioSpec Products, Bartlesville, OK, USA) for 30s. After homogenization, the lysate was transferred to a 2-mL microtube and centrifuged at 10,000 × *g* for 15 min at 4°C. The supernatant was collected, and samples were stored at -80◦C until analysis. The assay was performed as described by the manufacturer, with a standard curve of NADPH and samples in duplicate using a 96-well plate. The OD was measured at 340 nm at 0 and 30 min using a spectrophotometer (Varian Cary 50 UV-vis). The GPx activity is expressed in nmol/min/mL or mU/m, and one unit is defined as the amount of enzyme that will cause the oxidation of 1.0 µmol of NADPH to $NADP^+$ under the assay kit condition per minute at 25°C.

Statistical analysis

Statistical analysis was conducted using the MIXED procedure of SAS 9.4 (SAS Institute Cary NC, USA). Fixed effects were the treatments and run (as in replicate of same analysis in different days), while replicate within each run was considered as a random effect. Treatments were compared using orthogonal contrasts of (1) linear effect of CA (0, 3, 6, and 9 mg/L stimulated with 500 µM H_2O_2), and (2) quadratic effect of CA (0, 3, 6, and 9 mg/L stimulated with 500 µM H_2O_2). Data are shown as least squares means ± standard error of the mean (SEM) for cell viability and intracellular ROS, while GPx and SOD are displayed as fold change over culture medium control. Three replicate cultures were completed for SOD and GPx, while four replicate cultures were completed for intracellular ROS and Viability Assays. Significance was declared at $p \leq 0.05$ and tendencies as $p \leq 0.10$.

Results and discussion

This study tested the hypothesis that CA would have a protective effect on cultured cells subjected to hydrogen peroxide-induced oxidative damage. Results of cell viability after 12 h of stimulation with CA followed by 12 h of induction of oxidative damage are present in Figure 3.1A and Table 3.1. We observed a trend ($p = 0.10$) of a treatment effect on viability. As expected, the control has the highest cell viability. In comparison with control, hydrogen peroxide treatment reduced cell viability in all the treatments, except for CA 9 mg/L, which did not differ from control ($p = 0.30$).

The effects of CA on cell viability have been reported by others, using different cell lines, CA concentrations, and *in vitro* stressors. For example, Das et al. (2018)

observed a CA dose-dependent (1-4 µM) increase in cell viability in arsenic-induced toxicity of murine hepatocytes via the reduction of oxidative stress, MAPK activation, and programmed cell apoptosis. In addition, a study by Costa et al. (2007), using greater levels of CA (10, 20, and 30 µM), also showed a dose-dependent cytoprotective effect of CA when hepatocytes were exposed to aflatoxin B1, which was evident by the greater cell viability after 24 h of toxicity exposure. The authors attributed this effect to the reduction of intracellular ROS and DNA oxidation within the cell.

In contrast, CA has also been correlated with decreased cell viability in other studies. At concentrations of 70 µM, CA reduced hepatocyte (Hep G2 cells) viability to around 70% (Costa et al., 2007), while a 10 µM concentration was enough to inhibit the viability of AML12 cells as reported by Wang and Takikawa (2016). In the present study, the concentrations of CA applied were around 9, 18, and 27 µM for the 3, 6, and 9 mg/L treatments, respectively. At those concentrations, CA-treated cells exhibited cell viability similar to the oxidative damage positive control (500 µM H_2O_2). However, at 9 mg/L, CA increased the viability enough to be similar to the control treatment, suggesting a level of cytoprotective effect at this concentration for AML12 cells subjected to hydrogen peroxide-induced oxidative damage *in vitro*.

Results of intracellular ROS production after 12 h of stimulation with CA followed by 12 h of induction of oxidative damage are present in Figure 3.1B and Table 3.1. Contrary to our initial hypothesis and its reported properties as a ROS scavenger, CA treatment did not affect intracellular ROS production when AML12 cells were incubated with 500 µM H_2O_2, used to simulate oxidative damage *in vitro.* In contrast to our study,

Wang and Takikawa (2016) observed an inhibition in ROS production when hepatocytes were subjected to 3 mM of H_2O_2 in the presence of 1 μM of CA. According to these authors, CA likely protects against H_2O_2-induced liver damage by upregulation of SIRT1. The upregulation of SIRT1 expression by CA has also been reported by Teng et al. (2019), which observed an inhibition of neuronal apoptosis, and by Hu et al. (2015), who found a protective effect of CA against hydrogen peroxide-induced oxidative injury in hepatocytes. Since the SIRT1 signaling pathway is associated with the expression of antioxidants (e.g., catalase and SOD) via the FoxO pathways (Salminen et al., 2013), those results indicate that, other than being a direct ROS scavenger, CA affects oxidative status through activation of antioxidant pathways. In terms of the absence of protective effects of CA on ROS production in the present study, it should be considered that the H_2O_2 levels used were 6 times lower than those reported by Wang and Takikawa (2016) and Hu et al. (2015), which lead us to hypothesize that oxidative damage in the present study was not high enough to be influenced by the presence of CA. Moreover, the levels of CA applied by these other authors were lower compared with the present study. Wang and Takikawa (2016) used 1 μM CA, while Hu et al. (2015) used levels between 2.5 and 10 μM of CA, which suggest that the levels used in our study (9, 18, 27 μM for the 3, 6, and 9 mg/L treatments, respectively) might be excessive to promote a desirable physiological protection against H_2O_2.

The activity of the antioxidant enzymes SOD and GPx after 12 h of stimulation with CA followed by 12 h of induction of oxidative damage are present in Figure 3.2A and B, respectively, and Table 3.1. Glutathione peroxidase is a selenium-dependent

enzyme that catalyzes the degradation of hydrogen peroxide and organic peroxides to less reactive water and alcohols, while the different types of superoxide dismutases are copper, zinc, or manganese-containing enzymes that reduce superoxide anion (Sordillo and Raphael, 2013).

No effect of treatments on GPx activity in AML12 cells was observed. In contrast, we observed a treatment effect ($p = 0.03$) on SOD activity, in which CA treatments at 6 and 9 mg/L presented greater SOD activity when compared to Control ($p < 0.03$). A linear ($p < 0.01$) increase in SOD activity was observed when CA concentrations were increased from 0 to 9 mg/L. In contrast to our study, CA at 25 and 50 µM levels have been reported to increase GPx activity in lipid hydroperoxide-mediated oxidative stress in colorectal cells (Caco-2 Cells), while SOD activation was only observed when levels of CA reached 100 µM (Wijeratne and Cuppett, 2007). Moreover, Sahu et al. (2011) showed that CA at 100 mg/kg of BW enhanced the activities of GPx and SOD in rat kidneys exposed to toxic doses of cisplatin. Similarly, Xiang et al. (2013) reported that CA supplementation at 15, 30, and 60 mg/kg of BW markedly enhanced the cellular antioxidant defense system (e.g., superoxide dismutase, glutathione peroxidase, and glutathione) in serum and liver of rats after LPS challenge.

The positive effects of CA on the antioxidant mechanism might be attributed to its role in the activation of the PI3K/Akt/NRF2 signaling pathway (de Oliveira et al., 2015; de Oliveira et al., 2016) and through the SIRT1 pathway, as discussed previously. Nuclear factor erythroid-2 related factor 2 (NRF2) is a transcription factor involved in the cellular response to oxidative stress, inducing the expression of several protective

enzymes, such as glutathione peroxidase and superoxide dismutase (de Oliveira et al., 2016). Although in the conditions of the present study CA did not affect GPx activity, a dose-dependent effect on SOD activity might indicate an increase in the overall antioxidant capacity of AML12 subjected to hydrogen peroxide-induced oxidative cell damage.

Conclusions

The findings of the present study revealed that carnosic acid promotes positive effects on viability and antioxidant capacity through superoxide dismutase activity in mouse hepatocytes subjected to hydrogen peroxide-induced oxidative cell damage *in vitro*. Results from this study indicate that carnosic acid supplementation might be beneficial during periods of redox balance alterations *in vivo*, although further studies are necessary to determine dose-dependent responses and safety levels. In addition, future studies should address the effects of CA on NEFA-induced ROS production in bovine hepatocytes to simulate the oxidate stress damage caused to the liver during the transition period of dairy cows.

Table 3.1. Effect of carnosic acid (CA) on H_2O_2-induced AML12 cells on viability, intracellular ROS production, superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities.

Parameter	Treatments					SEM[1]	*p*-Value		
		500 µM H_2O_2 + CA					Trt[5]	Linear[6]	Quadratic[7]
	Control	0 mg/L	3 mg/L	6 mg/L	9 mg/L				
Viability[2]	5149.0[a]	4429.1[b]	4376.1[b]	4529.2[b]	4813.4[ab]	225.5	0.10	0.20	0.46
Intracellular ROS[3]	6.92[b]	9.25[ab]	10.08[a]	10.17[a]	11.25[a]	0.92	0.02	0.13	0.89
SOD activity[4]	1.00[c]	1.05[bc]	1.13[bc]	1.38[ab]	1.56[a]	0.13	0.03	<0.01	0.64
GPx activity[4]	1.00	1.05	1.40	1.19	1.01	0.31	0.81	0.80	0.36

[1] Largest standard error of the mean is shown.
[2] Luminescence measurement.
[3] Relative fluorescence units (RFU).
[4] Fold change over control.
[5] *p* value for the comparison between all treatments.
[6] p value for the linear effect of CA (0, 3, 6, and 9 mg/L stimulated with 500 µM H_2O_2).
[7] p value for the quadratic effect of CA (0, 3, 6, and 9 mg/L stimulated with 500 µM H_2O_2).

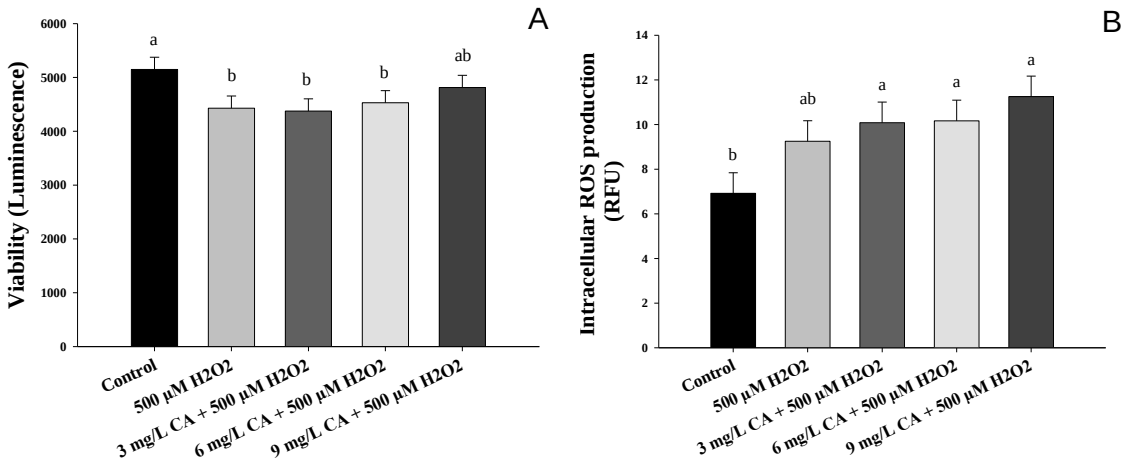

Figure 3.1. Effect of carnosic acid (CA) on H_2O_2-induced AML12 cells on Viability (A) and Intracellular ROS production (B). AML12 cells were pretreated with different concentrations of CA (3.0, 6.0 and 9.0 mg/L) for 12 h. Means with different letters (a–b) differ ($p < 0.05$). Four replications were done for each experiment. Values are means, with SEM represented by vertical error bars.

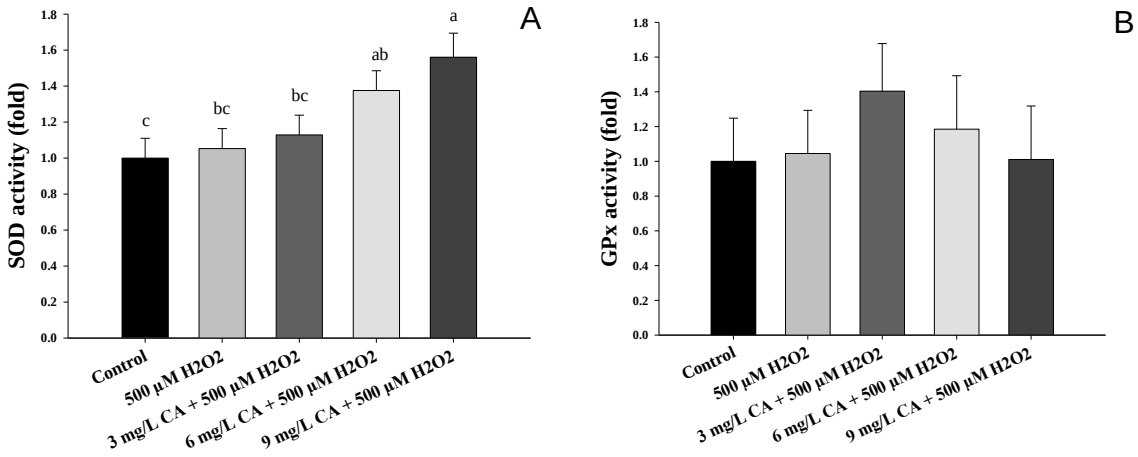

Figure 3.2. Effect of carnosic acid (CA) on H_2O_2-induced AML12 cells on Superoxide Dismutase (SOD) (A) and Glutathione Peroxidase (GPx) (B) activities. AML12 cells were pretreated with different concentrations of CA (3.0, 6.0 and 9.0 mg/L) for 12 h, and then treated with 500 µM H_2O_2 for 12 h. Means with different letters (a–c) differ ($p < 0.05$). Four replications were done for each experiment. Values are means, with SEM represented by vertical error bars.

CHAPTER 4. OVERALL DISCUSSION

The many challenges faced by transition dairy cows have been a topic of discussion and research for the last few decades. Although different strategies have been applied to reduce the severity and incidence of health disorders around parturition, this period remains the most critical in the dairy cow's life. Oxidative stress, an underlying factor for dysfunctional immune response and inflammation, has been studied as a key factor in the search for a better transition from pregnancy to lactation in high-yielding dairy cows. Most recently, natural compounds with antioxidant properties have been investigated as alternatives to commercial antioxidants (e.g., vitamins and minerals).

In this context, we proposed the use of carnosic acid, a phenolic diterpene found in plants of the Lamiaceae family, such as rosemary, known for its antioxidants, anti-inflammatory, and antimicrobial properties. To our knowledge, this is the first experiment to evaluate the effects of carnosic acid in transition dairy cows. Positive effects of carnosic acid infusion in the *in vivo* study regarding milk production and biomarkers of inflammation and oxidative stress led us to further investigate this natural compound in an *in vitro* study. The *in vitro* study, using mouse hepatocytes under oxidative stress conditions, showed interesting results as carnosic acid increased cell viability and superoxide dismutase activity under the experimental conditions, further proving its protective and antioxidant properties.

Although both *in vivo* and *in vitro* studies showed promising results, these experiments present some limitations. There are missing pieces of information as we

cannot yet determine the exact mechanism in which carnosic acid acts in the context of the transition cow metabolism. In the *in vivo* study, there was a limitation of low number of experimental animals. In addition, no differences were observed among treatments based on important oxidative stress blood biomarkers such as ROS, antioxidant capacity (e.g., ORAC and FRAP), and ratios between oxidants and antioxidant defenses (e.g., ROM/FRAP and ROM/ORAC). For the *in vitro* study, contrary to our hypothesis, carnosic acid neither reduced ROS concentration nor increased glutathione peroxidase activity in mouse hepatocytes under the proposed carnosic acid dosages and oxidative stress conditions.

As future perspectives for the use of carnosic acid or its plant-based source rosemary to transition dairy cows, future research should address a) how carnosic acid is metabolized in ruminants, b) estimate optimal dosages of supplementation, and c) which mechanisms might affect immune cells and hepatic metabolism of transition dairy cows.

As an example of a possible follow-up study, different levels of rosemary leaves could be supplemented from -21 to 21 days relative to calving to evaluate its effects on the antioxidant capacity and inflammatory status of transition cows following parturition. Concentrations of carnosic acid and other rosemary phenols could be determined in blood, milk, and peripheral tissues following supplementations to further understand its pharmacokinetics and metabolism. The effects of dietary rosemary leaves or extracts in the rumen microbiota and environment could also be addressed. In addition, liver tissue and PMNL gene expression during the period of supplementation might help to

determine the mechanism by which carnosic acid might have positive effects on milk production, redox balance, and inflammatory status, as observed in our *in vivo* study.

An important aspect to be considered when supplementing carnosic acid or its plant-based sources to dairy cows is the possible alternations in the milk. Increased concentration of carnosic acid in milk of goats supplemented with rosemary leaves was reported by Jordan et al. (2010). Moreover, these authors also observed an increase in plasma concentrations of carnosic acid of the suckling kids from supplemented goats. Results from this previous study raise questions regarding how carnosic acid supplementation in dairy cows might a) alter milk organoleptic properties, b) affect milk shelf life, and c) impact absorption and contribute to beneficial effects in humans.

Overall, the present studies show potential benefits of providing antioxidants such as carnosic acid to transition dairy cows in terms of milk production and blood biomarkers related to inflammation and oxidative stress. Based on our *in vitro* study, we observed that carnosic acid positively affected the antioxidant capacity of hepatocytes under oxidative damage conditions, which is a promising aspect considering the liver as a central organ during the peripartal period.

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