Is the NLRP3 inflammasome a potential biomarker to avoid the misuse of antibiotics of dairy cows during the transition period?

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SUMMARY

Currently, we know that there is a close relationship between the oxidative stress status and inflammatory processes during the transition period that severely endangers animal welfare and production. However, there are still certain aspects to be discovered, such as knowing if this inflammatory state is attributable to metabolic disturbances or infections. This information is important, because depending on the cause, the treatment varies, including the appropriate use of antibiotics.

Inflammasomes are multi-complex proteins that cause inflammation under both non-sterile and sterile conditions (the latter due to the presence of harmful substances). This review provides an update focused on the most studied inflammasome complex: the NLRP3 that plays a central role in several inflammatory conditions with metabolic origin. However, there is little information in relation to this protein activation in the pathogenesis of metabolic disturbances that appear in periparturient dairy cows. The latest research in this field suggests that this would be an important aspect in understanding the metabolic stress that characterizes the transition period in dairy cows. Thus, application of what is known about inflammasomes to preventive strategies that inhibit NLRP3 activation could be a research focus in a near future, contributing to differentiate when an animal has an inflammatory or infectious process, avoiding the misuse of antibiotics that are commonly employed to prevent potential uterine or mammary infections linked to an oxidative stress (OS) state in the cow. This review is in line with the new trends that explore new ways to reduce the misuse of antibiotics in dairy production.

KEY WORDS

NLRP3 inflammasome; oxidative stress, metabolic diseases; transition dairy cows.

INTRODUCTION

Mammals have two lines of defense when responding to an external antigen: the *innate* and the *adaptive* responses of the immune system. Inflammasomes are multi-complex proteins that cause inflammation under both non-sterile and sterile conditions. Thus, they are involved in the unspecific innate response. These inflammatory proteins are activated in response to a wide range of stimuli, including bacteria, bacterial toxins, and also some endogenous substances, such as cholesterol crystals, monosodium urate crystals, islet amyloid polypeptide, and other molecules such as the ATP released by damaged or dying cells[1].

In the last few years, the role of these multi-protein complexes in human metabolic syndromes has been the focus of relevant research[2]. Nevertheless, to the authors' knowledge, hitherto no previous study has considered the role of these proteins in the pathogenesis of metabolic syndromes in dairy cattle. Dairy cows experience a pro-inflammatory status around the time of calving[3], even in the absence of clinical disease[4]. Indeed, dysfunctional inflammation is now widely appreciated as a common underlying component of many metabolic diseases that affect periparturient dairy cattle. Therefore, a timely and natural resolution of inflammation is fundamental to overall dairy cattle health and well-being[5]. The discovery of the *inflammasome* protein complex in 2002 was a breakthrough in our understanding of how the immune system triggers inflammation. Now researchers are attempting to modulate its activity to treat disease[6].

Previous research showed that the NLRP3 inflammasome is responsible for *sterile inflammation* in humans and murine experimental models[1]. Since this inflammatory process is observed in periparturient dairy cows, this review will focus on the comprehension of inflammasome activation - especially NLRP3 - in periparturient dairy cows and its health implications.

As we mentioned above most of the studies in this area have been performed either in humans or murine models during gestation, and therefore the results might not be directly appropriate to cattle, but constitute a reference point. Thus, the aim of this review is to encourage future research on the role and potential uses of NLRP3 in controlling these inflammatory processes. Dysregulated inflammation leads to immunosuppression and increased disease risk in cattle. Hence, controlling the inflammatory processes where NLRP3 inflammasome is involved could contribute to a reduction in antimicrobial usage on farms and the development of antimicrobial-resistant bacteria.

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The role of inflammasomes in the innate immunity response

When the innate immune system is activated, antigen-presenting cells (APCs) and dendritic cells transmit the information about the stimuli to B and T lymphocytes. On the other hand, different populations of innate immune cells detect pathogens with a fixed number of germline-encoded *pattern recognition receptors* (PRRs)[6], located on the cell surface, endosomal membrane, and in the cytoplasm[7]. These PRRs are classified into two classes according to their subcellular location: 1) *Toll-like receptors* (TLRs) and *C-type lectin receptors* (CLRs), transmembrane proteins located in the cellular membrane and endosomes; and 2) other types of PRRs that are located in intracellular compartments such as the *RIG-I-like receptor* (RLR), *the AIM2-like receptor* (ALR), and the *nucleotide-binding domain and leucine-rich repeat-containing proteins* (NLR).

These PRRs recognize distinct *pathogen-associated molecular patterns* (PAMPs) found in microbes, allowing the identification of pathogens in tissues where these organisms should not be present. However, PRRs also recognize host-derived signals, the so-called *damage (or danger)-associated molecular patterns* (DAMPs). These are released as a result of tissue perturbations caused not only by germs but also by non-microbial insults, allowing general sensing of *stressed tissue*[1].

Following the identification of DAMPs or PAMPs by PPRs, several signaling cascades that promote gene transcription are activated[6]. These signaling cascades include nuclear factor-κB (NF-κB), activator protein 1 (AP1), and interferon regulatory factors (IRFs), which, in turn, increase the transcription of specific genes encoding cytokines, interferons, and other microbicidal or proinflammatory proteins. These proteins set up the cytosolic protein complexes called *inflammasomes* to activate proinflammatory caspase-1 and caspase-11. Rapid conversion of procaspase zymogens into enzymatically active proteases results in the production of proinflammatory IL-1β and IL-18 and the development of an inflammatory process called *non-sterile inflammation* due to microbial stimuli. This form of inflammation leads to the death of the affected cells by a process called *pyroptosis,* which combines characteristics of both apoptosis and necrosis and that passively releases cytoplasmic proteins (*alarmins*) to alert neighboring cells[1,8-11].

Conversely, s*terile inflammation* occurs when DAMP-triggered inflammation takes place in the absence of any foreign pathogens. Reviewing all the mechanisms implicated in both types of inflammation is beyond the scope of this article and the readers are directed to the review by Guo *et al.*[1].

Inflammasome formation

Inflammasomes are a group of proteins that consist of three components: (1) a cytoplasmic sensor molecule, (2) caspase-1 or caspase-11, and (3) frequently, the adaptor protein ASC[12]. There are fundamental differences between inflammasomes depending on the stimuli that trigger the activation. Figure 1 shows a schematic overview of activation mechanisms of the most studied inflammasomes.

Each protein has a different range of activation signals, although it may receive overlapping signals. The NLRC4 and AIM2 inflammasomes can be activated by specific PAMPs or specific bacterial proteins; while the NLRP3 inflammasome is activated by PAMPs but also DAMPs[8,13].

The focus of this review is the NLRP3 inflammasome, due to its link to metabolically triggered inflammation[2]. Readers are encouraged to consult previous publications regarding the role of other inflammasomes linked to inflammation of different origin (e.g., microbial infection[1,6,14]).

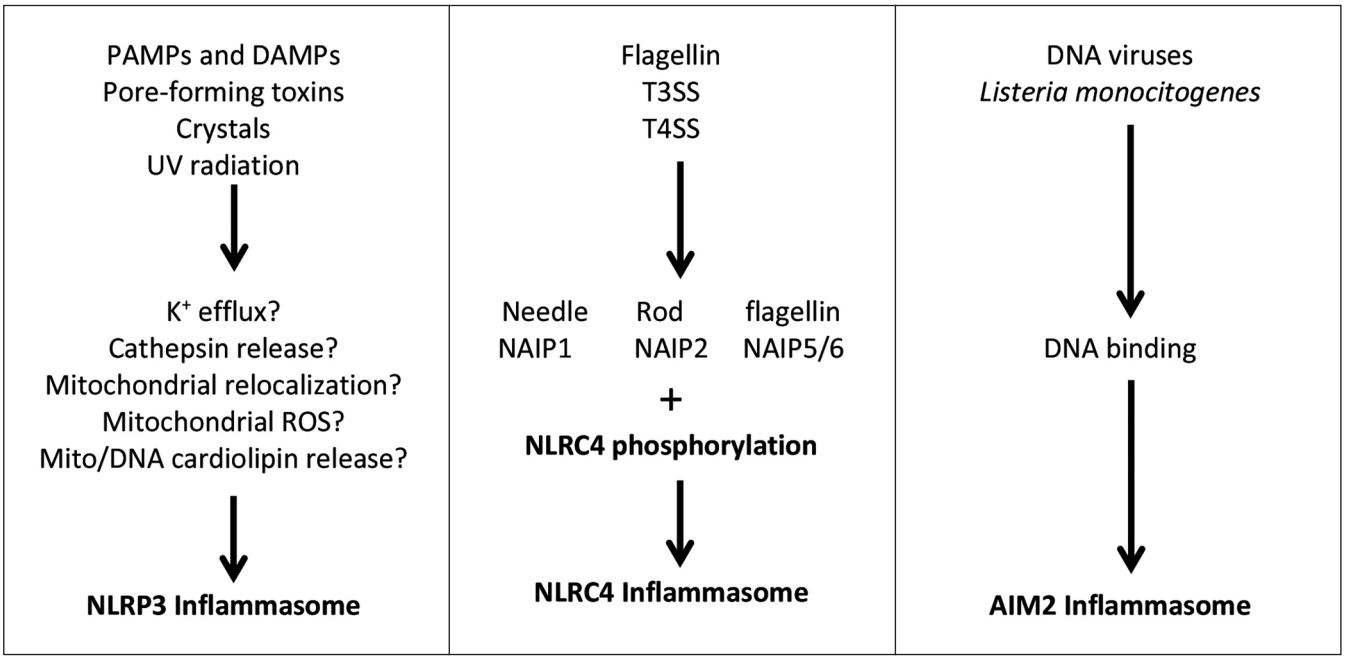

Figure 1 - Schematic overview of the proposed inflammasomes activation mechanisms[6]. The different inflammasomes recruit and activate caspase-1 in response to a variety of triggers. PAMPs, DAMPs, pore-forming toxins, crystals, and UV radiation are thought to activate the NLRP3 inflammasome by reducing intracellular K+ concentrations, by promoting cytosolic release of lysosomal cathepsins, by relocating NLRP3 to the mitochondrial outer membrane, and by inducing mitochondrial damage, which may be sensed by NLRP3 via the production of ROS or the cytosolic release of oxidized mitochondrial DNA and cardiolipin. Cells exposed to bacteria expressing flagellin or a type III (T3SS) or IV (T4SS) secretion system indirectly activate the NLRC4 inflammasome through NAIP proteins. AIM2 is activated by the presence of dsDNA in the cytosol of cells infected with Listeria monocytogenes and the DNA viruses cytomegalovirus.

The NLRP3 inflammasome

Within the inflammasomes, the NLRP3 is an approximately 700 kD polyprotein complex widely studied[15]. It is involved in the immune response resulting from the release of harmful substances by damaged host cells, causing *sterile inflammation*[2,8,16-17]. Activation of the inflammasome during infection can be protective, but unregulated NLRP3 inflammasome activation in response to non-pathogenic endogenous or exogenous stimuli can lead to unintended pathology[18-19].

The mechanisms of NLRP3 activation include: 1) potassium efflux out of the cell; 2) the generation of mitochondrial reactive oxygen species (ROS); 3) the translocation of NLRP3 to the mitochondria; 4) the release of mitochondrial DNA or cardiolipin; and 5) the release of cathepsins into the cytosol after lysosomal destabilization[6,14,20]. Different studies have suggested that metabolic disturbances are sensed inside the cell by organelles, such as endoplasmic reticulum or mitochondria, whose subsequent dysfunction, in turn, triggers a network of stress-induced signals that finally disrupt metabolic homeostasis[15,18,21].

Thus, metabolic diseases characterized by ROS overproduction are able to activate the NLRP3 inflammasome and to cause a sterile inflammatory status. Figure 2 offers a schematic overview of the NLRP3 inflammasome activation mechanisms.

In addition to ROS, there are a variety of other stimuli that can act as NLRP3 agonists. These include the presence of ATP, pore-forming toxins, crystalline substances (e.g., monosodium urate and calcium phosphate), nucleic acids, hyaluronan, uric acid, oxalate crystals, and hydroxyapatite crystals among others[22-23].

Inflammasome propagation

Phosphorylation of apoptosis-associated speck-like protein containing caspase recruitment (ASC), mediated by the kinases Syk and JNK, is the key point in the redistribution on inflammasome activation from the nucleus to the cytosol forming a large perinuclear aggregate in cells, called *ASC specks*. ASC specks released from affected cells lead to cleavage of extracellular pro-IL-1β and activation of caspase-1 in macrophages, internalizing the specks[1]. Thus, phagocytosis of extracellular ASC specks induces lysosomal disruption and results in an inflammatory response[18].

Assessment of NLRP3 inflammasome activation

The expression of different inflammasomes has largely been studied in innate immune cells such as macrophages. The biomarkers of their activation are the secretion of the mature forms of caspase-1 and IL-1β from these cells[12] (Figure 2). In some cases, the inflammasome activation has been determined by measuring the IL-1β and caspase-1 expression, via RT-PCR analysis. However, in the case of NLRP3, the more correct analytical methods are the identification of NLRP3 specks and the cleavage of caspase-1 or IL-1β by Western blot, or the release of IL-1β by ELISA[24-25]. On the other hand,

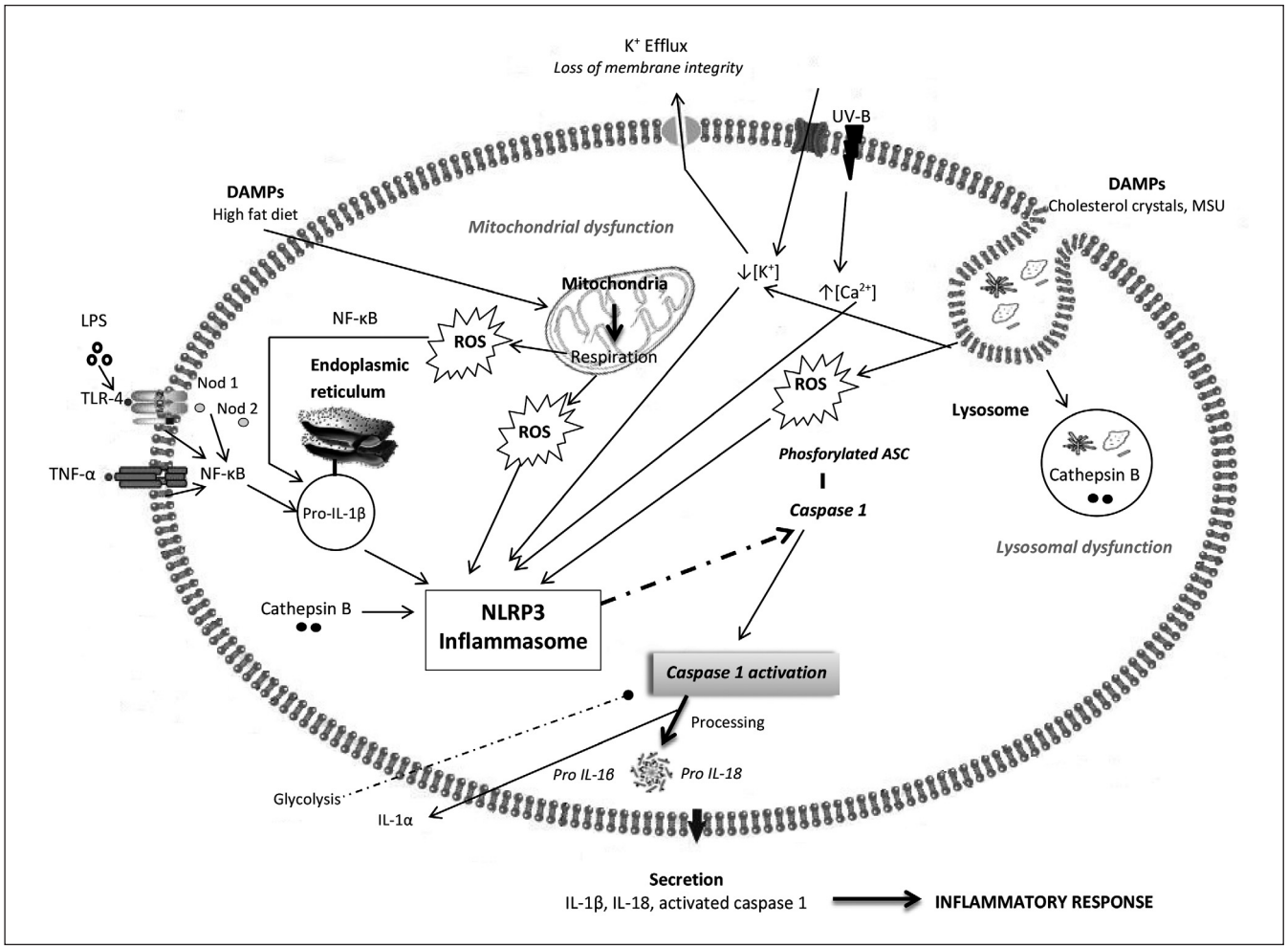

Figure 2 - NLRP3 inflammasome activation and signaling pathways (adapted from Bio-Connect BV, http://www.bio-connect.nl).

for the detection and cell distribution analysis of inflammasomes, the use of standard confocal microscopy combined with fluorescent tagging is required. This system uses mirrors to focus light on a sample at multiple points and depths, building a three-dimensional image. When fluorescent molecules are attached to inflammasomes, these can be identified inside the cells[10].

Regarding the analysis of inflammasomes at the protein level, ELISA and Western blot techniques have been widely used for the quantification of 1) caspase-1 (p10 or p20); 2) inflammasome signaling for NLRP3; 3) inflammasome activators; and 4) inflammasome priming activators. Most of these assessments have been performed using cell culture supernatant, but currently, there are commercial kits that allow these determinations using serum or plasma and colorimetric methods, which facilitate laboratory analysis[12]. Each method has benefits and disadvantages that should be considered carefully by the investigators. Reviewing the considerations regarding these different procedures and the methods for sample handling and storage is beyond the scope of this review. Readers are directed to previous publications in this area[12,24-26].

THE POTENTIAL ROLE OF NLRP3 INFLAMMASOME AS CLINICAL BIOMARKER OF METABOLIC DISEASES IN PERIPARTURIENT DAIRY COWS

In the case of dairy cattle, it is well known that the transition from the gestational non-lactating to the lactating state is characterized by significant physiologic and immunologic changes. As result of these changes, the animals suffer what is called *metabolic stress*, defined as an imbalance in the physiological homeostasis of an organism as a consequence of aberrant nutrient utilisation with excessive lipomobilisation, immune and inflammatory dysfunction, and an excessive ROS production, with great impact in the productive efficiency of the herd[3,27]. To date, there have been numerous studies to better understand the underlying causes of metabolic diseases around the time of parturition in order to design more effective management practices to reduce transition cow health disorders[28].

During this time of increased metabolic activity, the production of ROS can exceed the neutralizing capacity of antioxidants, causing and oxidative stress (OS) status[29]. In this scenario, ROS activate the nuclear factor κB pathway (NF-κB; Figure 2), promoting inflammation[5,30].

On the other hand, during the transition period, there is a marked increase in energy demands for fetal growth and onset of lactation parallel to a reduction in dry matter intake and resulting in the development of negative energy balance (NEB[31]). In this situation, the intense lipomobilisation produces an increased flow of non-esterified fatty acids (NEFAs) into the liver. Inside the liver, NEFAs can follow three main paths: 1) they can be completely metabolized via β-oxidation for energy production through the tricarboxylic cycle, 2) partially oxidized to produce ketone bodies (acetone, acetoacetic acid, and beta-hydroxybutyric acid - BHBA), or 3) converted into triacylglycerols (TAGs). Usually, TAGs are packaged into very low-density lipoproteins for transport back to the adipose tissue or the mammary gland. However, the transport capacity of these lipoproteins is very limited in cattle. Therefore, this pathway is easily overwhelmed and the TAGs are stored within the hepatocytes, causing the condition commonly referred to as *fatty liver*[32]. Although the certain concentration of NEFAs and BHBA in blood is part of a normal adaptation to NEB in early lactation, excessive concentrations of NEFAs or BHBA indicate an excess of NEB which has been associated with detrimental health and production outcomes[33].

The main consequence of this excessive NEB is the impairment of the normal hepatic function, resulting in the development of *fatty liver syndrome*[34]. In murine models with similar conditions, it has been demonstrated that hepatocytes upregulate NLRP3 expression on activation of TLR4 signaling; moreover, Kupffer cells and sinusoidal endothelial cells express high levels of NLRP3[2]. Hence, the role of the NLRP3 inflammasome on hepatic dysfunction around the time of calving in dairy cows merits further research.

This metabolic status is complicated further by the fact that in this period has been registered status of decreased insulin sensitivity to prioritize the insulin-independent uptake of glucose by the mammary gland. This results in an increased expression of tumor necrosis factor (TNFα) that exacerbates the inflammatory response of the cows[35]. In fact, TNF appears to be the most potent priming stimulus for myeloid cell NLRP3 responses and is chronically elevated in many sterile inflammatory diseases whose pathology is worsened by uncontrolled NLRP3 inflammasome activation[11] probably due to the exposure of macrophages membrane mitochondrial damage, that increases mitochondrial ROS[36].

In addition, adipose tissue is not only a storage site for fat but also is a metabolically active tissue that synthesizes and secretes a vast array of biologically active substances, such as lipopolysaccharides (LPS), involved in regulating inflammatory responses[1]. Also, activated macrophages located within adipose stores produce cytokines and bioactive lipid mediators that are the same molecules that immune and inflammatory cells typically produce following pathogen stimulation[27]. Similarly, there are resident populations of leukocytes in all metabolic tissues and their presence suggests that these tissues are ready to respond to bioactive molecules that function in both metabolic and immunologic roles[21]. More recently, Sordillo[5] point out the role of *oxylipids* in dairy cows as a key point in the inflammatory response in periparturient dairy cows. In fact, changes in lipid metabolism in dairy cows around parturition can profoundly change the composition and concentration of these compounds and may be responsible for dysfunctional inflammatory responses during this time.

Furthermore, the study of Ster *et al.*[33] stated that elevated concentrations of NEFAs and BHBA are detrimental to immune function. Although dairy cows experience a pro-inflammatory status during the transition period that aids in the facilitation of parturition and plays a role in homeorhetic adaptations to onset of lactation[3,37-38], an excess of dysregulated inflammation predisposes dairy cows to metabolic diseases among other processes[34].

Therefore, the implication of NLRP3 inflammasome activation in response to a wide array of pro-inflammatory DAMPs generated under NEB opens new lines of research about the role played by NEFA and BHBA in the sterile in-

flammatory process. Until now, and in many cases, some of these inflammatory conditions finally resulted in infections due to the disruption of the host defense mechanisms, and have been treated using antimicrobials. The use and/or misuse of antibiotics in dairy farms, however, constitutes a worldwide concern and therefore an increased understanding of the inflammatory processes that take place in these animals will be beneficial in decreasing antimicrobial usage on farms. For this reason, deep research about the NLRP3 response in the metabolic stress associated with the transition period could be an interesting diagnostic tool, because NLRP3 inflammasome response to microbial versus sterile priming is not yet deeply studied. In fact, how exactly these different stimuli activate NLRP3 (presumably via a common downstream mediator,) is an area of extensive research elsewhere[39].

According to this item, a recent study[11] in murine models tested NLRP3 inflammasome responses to microbial versus sterile signals. Authors stimulated murine bone marrow-derived macrophages with a microbial (LPS and nigericin) or sterile (TNF and ATP) signals. Using ELISA and Western blot techniques found that sterile signals produced weaker macrophage NLRP3 inflammasome responses relative to microbial signals. Specifically, NLRP3 inflammasome responses (caspase-1 cleavage, cell death, and IL-1β secretion) were weakest when signals were sterile, with delayed kinetics relative to microbial signals. Their data could contribute to differentiating when an animal has an inflammatory or infectious process, avoiding the misuse of antibiotics that are commonly employed to prevent potential uterine, mammary or foot infections and that are usually linked to an OS state in the cow.

Nevertheless, according to Strowig *et al.*[8], not only is important the knowledge of inflammasomes activation but the final goal should be to identify inhibitory mechanisms. Research in human medicine has proposed several methods to achieve this and could be adapted for food-producing animals. For example, inhibition of SyK and JNK kinases prevents ASC speck formation and blocks caspase-1 activation, suggesting that this fact might have potential therapeutic use against inflammatory diseases[6,25].

Conversely, other practices are currently common on dairy farms to attempt to control dysregulated inflammation. For example, antioxidant supplementation during the periparturn period is beneficial for dairy cow's health, influencing different endogenous regulatory antioxidant mechanisms and counteracting the harmful effect of excess ROS production[29]. Among these regulatory mechanisms, Abuelo *et al.*[35] pointed out the nuclear factor E2-related factor 2 (Nrf2), a redox-sensitive factor that controls the transcription of genes encoding various antioxidative and cytoprotective proteins. The Nrf2 is anti-inflammatory and their action is associated with inhibition of the NF-κB pathway and inhibition of proinflammatory cytokine production that causes NLRP3 activation in cases of excessive ROS production. Indeed, Nrf2 factor is activated as an adaptive response defense mechanism against OS. For example, inflammation is commonly observed in those chemically-induced pathologies with Nrf2 deficiency[40]. Nevertheless, excessive antioxidant supplementation could also impair the above-mentioned mechanism, as it could decrease the expression of genes encoding antioxidative molecules thereby dysregulating redox balance[29]. Taking into account that several aspects about the biochemical events underlying the connection between Nrf2 and NLRP3 remain unclear, studies that jointly evaluate OS, antioxidant supplementation, and NLRP3 activation could provide useful information about how much antioxidant is adequate, establishing an accurate protocol for supplementation.

The use of natural plant extracts as preventive and treatment coadjuvants of different inflammatory status has been considered. Red ginseng extract inhibits NLRP3 inflammasome activation[40], Indeed, a component of the ginsenosides family, attenuates dsDNA-induced IL-1β secretion. In general, ginsenosides show anti-inflammatory properties by inhibiting NF-κB activation in association with reduced secretion and/or mRNA expression of proinflammatory mediators[41]. On the other hand, *Sophora flavescens*, *Lycium barbarum*, *Impatiens textori*, *Syneilesis palmata*, *Aloe vera*, citral (3,7-dimethyl-2,6-octadienal), celastrol, sulforaphane, schisandrin, resveratrol, dehydrodiconiferyl alcohol, luteoloside, *Pulsatilla decoction*, and *Wuling San* have been reported to suppress the function of the NLRP3 inflammasome. According to our experience, medicinal plants and their derivatives can be useful for inflammation-related disorders by suppressing NLRP3 inflammasome activation[42]. Hence, the use of natural plant extracts could also be a useful and feasible tool to control sterile inflammatory conditions in dairy cows.

CONCLUSION

Inflammation is a critical component of various metabolic diseases in mammals. The application of the recent discoveries about the role of inflammasomes in human metabolic diseases to veterinary medicine open new lines of research to enhance animal health. Although this review hypothesizes about the usefulness of the application of the discoveries made in human medicine on this inflammasome, we believe that its application in veterinary medicine can contribute to determining septic of non-septic inflammatory states, thus contributing to optimize the use of antibiotics. In addition, the activation of the NLRP3 inflammasome results in humans in an aseptic inflammatory response similar to what is observed in periparturient dairy cattle. Hence, future studies directed to investigating the role of the NLRP3 inflammasome in cattle disorders around calving could provide new insights into innovative ways for correcting and preventing dysregulated inflammation and its adverse effect on the health and productivity of dairy cows.

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