http://dx.doi.org/10.1111/jpn.12273

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Abuelo, A., Hernandez, J., Benedito, J.L., Castillo, C., 2014. The importance of the oxidative status of dairy cattle in the periparturient period: revisiting antioxidant supplementation. Journal of Animal Physiology and Animal Nutrition [doi: 10.1111/jpn.12273].

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Review article

The importance of the oxidative status of dairy cattle in the periparturient period: Revisiting antioxidant supplementation.

Ángel Abuelo[1], Joaquín Hernández, José L. Benedito and Cristina Castillo

Department of Animal Pathology. College of Veterinary Medicine. University of Santiago de Compostela. Campus Universitario s/n. 27002 – Lugo (Spain).

Running head: Antioxidant therapy in periparturient dairy cattle

[1] Correspondence: *Dr. Ángel Abuelo: angel.abuelo@usc.es; angel.abuelo.sebio@gmail.com. Phone: (+34) 982822643. Fax: (+34) 982822627*

http://dx.doi.org/10.1111/jpn.12273

Summary

Dairy cows are especially vulnerable to health disorders during the transition period, when they shift from late pregnancy to the onset of lactation. Diseases at this stage affect not only the animals' wellbeing, but also cause a major economic impact in dairy farms, because apart from treatment costs, affected cows will not reach their peak milk-producing capacity. The overproduction of reactive oxygen species (ROS) leads to oxidative stress, which has been identified as an underlying factor of dysfunctional inflammatory responses. Supplementation with vitamins and trace elements attempts to minimize the harmful consequences of excessive ROS production, thereby trying to improve animals' health status and to reduce disease incidence. However, results regarding the effects of supplementing antioxidants on dairy cows' health and performance have been inconsistent, because in most cases the antioxidant potential of the animals was not assessed beforehand and the nutritional strategy planned accordingly. Therefore, reviewing the physiological and harmful effects of ROS production, along with the different options available for assessing the redox balance in dairy cattle and some of the key findings of different supplementation trials, could bring one step forward the on-farm application of determinations of oxidative status for establishing nutritional strategies early enough in the dry period that could improve transition cow health.

Keywords: Oxidative stress; Transition period; Dairy cow; Vitamins; Trace elements.

1.- Introduction

The transition from gestation to lactation, namely 'transition period', is the most critical moment in the lactating cycle of dairy cows and entails the last 3 weeks before and the first 3 weeks after calving. This period is characterized by several orchestrated metabolic and endocrine changes as a consequence of the increased nutrient demands that aim to support the milk production (Bell 1995). Parallel to these changes in demands, dairy cattle undergo around calving a period of dysfunctional immune response activity that last several weeks postpartum (Kehrli et al. 1989; Goff and Horst 1997). Indeed, reduced cell immunity has been associated with diseases such as retained placenta, metritis or mastitis (Cai et al. 1994). Therefore it is not surprising that approximately 75% of disease incidence (mastitis, metritis, ketosis, displacements of the abomasum, etc.) within herds occurs within the first month of lactation (LeBlanc et al. 2006), with the period of maximal risk of infectious and metabolic disorders being the first 10 days after calving (Ingvartsen et al. 2003), overlapping with the greatest acceleration of milk yield.

This period of increased metabolic activity implies an increase in the production of free radicals, which are produced in the mitochondria as a normal by-product of cellular respiration at the electron transport chain reaction (Halliwell and Gutteridge 2007). Changes in the oxidative metabolism occur in the transition period, and several studies suggested that oxidative stress increases the susceptibility of dairy cattle to diseases (Bernabucci et al. 2005; Castillo et al. 2005; 2006; Sordillo and Aitken 2009).

The practice of supplementing animals with vitamins and trace elements attempts to minimize the harmful effects of excessive free radicals' production (Politis 2012), improving animals' health status and reducing disease incidence (Bourne et al. 2008). However, the systems used to calculate nutrient requirements of dairy cattle (i.e. NRC, INRA) have traditionally estimated the needs of vitamins and trace elements for maintenance, growth, pregnancy, and lactation considering the prevention of deficiency pathologies. This approach has been recently criticized (Overton and Yasui 2014) because (i) the NRC (2001) underestimates the requirements of dairy cattle for some trace elements, and (ii) research has shown that supplementation slightly above requirement levels improves animals' health and performance.

Nevertheless, there is also evidence that excessive supplementation has some detrimental effects, such as increased incidence of mastitis (Bouwstra et al. 2010b; Bouwstra et al. 2010a), because an excess of antioxidants can result in an increase in the production of pro-oxidants (Rizzo et al. 2013). Therefore this review will consider some of the physiological functions of free radicals within the organism and the harmful consequences of their excessive production, alongside with strategies for their prevention and methods for assessment of the redox balance.

2.- Functions of oxidants and consequences of Oxidative stress

Oxidants are substances that are able to reduce themselves and oxidize other molecules. Free radicals are oxidizing agents, characteristic of atoms or molecules, which have in their outer shell one or more unpaired electrons

(Maxwell 1995; Halliwell and Gutteridge 2007); however, not only radicals are oxidant substances, but also other molecules that without having unpaired electrons can lead to the formation of radicals, i.e. hydrogen peroxide, singlet oxygen, peroxynitrite, etc. Oxidants can be classified into two categories: reactive oxygen species (ROS) and reactive nitrogen species (RNS). ROS are the most abundant free radicals in biological systems (Miller et al. 1993a), therefore when generally speaking, RNS (such as nitroxide, peroxynitrite, nitrogen dioxide, dinitrogen trioxide, etc.) are ascribed to ROS.

ROS are formed normally as by-products of cellular metabolism, and not only are essential for physiological processes like cell differentiation or proliferation (Halliwell and Gutteridge 2007), but also play a key role in the host immune response; since ROS generation is involved in the destruction of phagocyted pathogens and also because of their involvement in the expression of cell signalling molecules like cytokines, eicosanoids and other immunoregulatory substances (Forman and Torres 2002; Asehnoune et al. 2004) that are indispensable for a satisfactory response against the invading pathogens; besides, ROS are essential for optimizing inflammatory responses (Kvietys and Granger 2012).

However, when produced in excess, ROS can harm the cells, leading to loss of cell function and tissue damage (Valko et al. 2007; Sordillo and Aitken 2009). Luckily, the organism is equipped with several antioxidant substances that are capable of counteracting overproduction of ROS and their accumulation. Nevertheless, when the production of ROS overwhelms the neutralizing capacity of antioxidants, a condition named Oxidative Stress (OS) develops. This imbalance can result in tissue damage as a result of the oxidation of DNA, cellular proteins and lipids. As an example, if a membrane phospholipid is peroxidised, lipid radicals are formatted; and if not eliminated by antioxidants, they can stimulate the oxidation of adjacent membrane fatty acids, entering onto a vicious cycle that results in the loss of membrane function, and even cell death if the condition is not fixed (Halliwell 2007; Lykkesfeldt and Svendsen 2007).

3.- Oxidative stress at the transition period: Implications for animal health

The transition period was defined by Grummer (1995) as the period from 3 weeks pre-calving until 3 weeks post-calving. Several review articles have summarized the important physiological and immunological changes that take place as the cow moves from a non-lactating state to the onset of lactation (Grummer 1995; Drackley 1999; Ingvartsen et al. 2003). This period is associated with an increased incidence of production diseases; and as the effects of these diseases on dairy cow health and productivity extend far into the following lactation (Mulligan and Doherty 2008); therefore successful adaptation of dairy cows during this period is vital for ensuring efficiency in the dairy industry.

When a cow fails to adapt to the increasing demands of the transition period (foetal growth, calving, and onset of lactation), metabolic stress results; which can be broadly defined as the hypermetabolic, catabolic response to an imbalance in physiological homeostasis, and it is usually caused by a decreased amount of glucose in the bloodstream. Hypoglycaemia is a common feature in fresh cows, as almost all the glucose is taken up by the udder for lactose synthesis, and therefore a dysregulation in insulin response develops in order to prioritize the use of glucose by the mammary gland (De Koster and Opsomer 2013), which takes place insulin independently. As the energy requirements of the increasing milk yield are not met by diet in the first weeks after calving, the cow must use its own energy reserves. Thus, lipid mobilization is a typical characteristic of this state of negative energy balance (NEB). As a consequence of this, non-esterified fatty acids (NEFA) are released into the bloodstream and used as an energy source. Cows successfully adapt to NEB when the release of NEFA is limited to concentrations that can be fully metabolized for energy needs associated with gluconeogenesis (Sordillo and Raphael 2013). If the concentrations of NEFA exceed the processing capacity of hepatocytes, like during excessive lipid mobilization, then the liver function is impaired by triglyceride accumulation –causing fatty liver- and ketone bodies, such as β-hydroxybutyrate (BHB), overproduced.

Increased blood NEFA concentrations impact inflammatory responses of transition cows (Sordillo and Raphael 2013), and when used as energy substrate at the peripheral tissues, NEFA enhance reactive oxygen species (ROS) production during β-oxidation (Schönfeld and Wojtczak 2008). Oxidative stress can cause extra lipolysis, thereby contributing to higher NEFA levels in periparturient cows (Sordillo and Raphael 2013), entering in a vicious cycle of lipolysis and ROS production (Figure 2). High NEFA concentrations and ROS production are characteristics of metabolic stress and have been recognized as risk factors for diseases in transition cows, such as mastitis, retained foetal membranes, ketosis and fatty liver (Herdt 2000; Sordillo and Raphael 2013).

OS is a relatively new field of research in ruminants' medicine (Celi 2011a), but it is also thought to be a significant underlying factor in dysfunctional host immune and inflammatory responses, which can increase cows' susceptibility to health disorders (Sordillo and Aitken 2009). Animals experiencing higher inflammatory response show a lower daily milk yield, poorer fertility and a greater disease incidence (Bertoni et al. 2008; Trevisi et al. 2012). Indeed, treatments with anti-inflammatory agents after calving showed an improved health status and performance (Bertoni et al. 2004; Farney et al. 2013b). However, recent research has shown that a certain degree of inflammation is not only a common feature after calving, but is an adaptive rather than pathological process, since some level of inflammation is actually required or beneficial to milk production and for successful transition period adaptation (Farney et al. 2013a; Yuan et al. 2013). Therefore the debate continues about what amount of OS and/or inflammation is required for physiological processes and what amount costs animal performance. The ability of establishing a threshold for this will help establishing preventive and corrective measures.

As dysfunctional inflammation is believed to be the nexus between the increased incidence of infectious and metabolic health disorders (Sordillo and Raphael 2013), a better understanding of the impact of how these adaptive processes impact metabolic stress will be helpful for stablishing control strategies for the prevention of periparturient cattle diseases; and in this context, oxidative stress might be the nexus between nutrient metabolism and inflammation in dairy cows around the time of calving (Sordillo and Mavangira 2014).

4.- Assessment of the oxidative status of dairy cows

OS is well-established as an underlying cause of both infectious and metabolic diseases but as no specific clinical symptoms are shown, special analytical methods are needed to detect and alleviate deleterious impact of OS on transition cow health (Celi 2011a). As the concept of OS is defined by an imbalance between pro- and antioxidants (Figure 1), methods for the evaluation of OS usually entail the quantification of both pro- and antioxidants (Celi 2011b; a). Several methodologies have been developed for these purposes, each with its own advantages and disadvantages. However, it is beyond the aim of this review to report and compare the different methods available, which has been already published elsewhere (Celi 2011a); and therefore here only a brief description of the key features of oxidative status assessment is reported.

4.1.- Pro-oxidants

Electron spin resonance is considered the 'gold standard' for the determination of the oxidant status; however this technique entails complex procedures that are not easily found in most laboratories and therefore is not considered suitable for routine analysis (Celi 2011a) and has only been anecdotally applied in bovine research (Gaál et al. 2006). Fortunately, a kit for the quantification of ROS in biological fluids has been developed and validated (Alberti et al. 2000), allowing their determination through spectrometry, a technique commonly available in most diagnostic laboratories. As ROS is a collective term that includes not only oxygen-centred free radicals, but also some non-radical derivates of oxygen, such as hypochlorous acid or hydrogen peroxide (Reilly et al. 1991), the plasma concentration of ROS is considered an indicator of free radical production (Miller et al. 1993a), and has been reported in several studies regarding the redox status of cattle (Bernabucci et al. 2002; 2005; Dobbelaar et al. 2010; Pedernera et al. 2010; Castillo et al. 2012; Abuelo et al. 2013; Rizzo et al. 2013; Abuelo et al. 2014).

Nevertheless, other methods have been employed to estimate the production of ROS through the quantification of end-products of the radicals' attack to macromolecules. For example, advanced oxidation protein products (AOPP) are markers of oxidized proteins (Witko-Sarsat et al. 1996) that have been associated with several diseases in humans and with impaired reproduction in dairy cattle (Celi et al. 2011; 2012). However, further studies are still needed in veterinary medicine to unravel the implication of these substances in cattle diseases.

Likewise, lipids are prone to oxidation, and malondialdehyde (MDA) is generated as a consequence of lipid peroxidation, and as such is assayed as a biomarker of oxidative stress. Several methodologies are available for the quantification of MDA, but among them the thiobarbituric acid reactive substances (TBARS) (Janero 1990) is the most commonly encountered in the veterinary literature (Bernabucci et al. 2002; Gabai et al. 2004; Bernabucci et al. 2005; Wullepit et al. 2009; Tanaka et al. 2011; Wullepit et al. 2012). This spectrophotometry method is based on the capacity of MDA to react with thiobarbituric acid to produce a red pigment. However, it is noteworthy that MDA determination has been criticized for its low specificity and artefact formation (Celi 2011a), besides TBARS are considered inaccurate, as they detect a wide range of lipid peroxidation products and are not specific for MDA (Halliwell and

Chirico 1993). These facts might be behind some of the controversial findings of early studies on oxidative stress around the time of calving (Celi 2011a).

4.2.- Antioxidants

The organisms are well-equipped with a network of substances capable to counteract the oxidative attack. Endogenous antioxidants have been traditionally classified into 3 different groups (Miller et al. 1993a): (i) enzymatic antioxidants -being glutathione peroxidase (GSH-Px; EC 1.11.1.9) and superoxide dismutase (SOD; EC 1.15.1.1) the most widely known ones-, (ii) nonenzymatic protein antioxidants and (iii) nonenzymatic low-molecular-weight antioxidants. A detailed description of the activity of each antioxidant goes beyond the purpose of this review, and is available elsewhere (Celi 2011a).

Quantification of antioxidant substances separately does not provide a good image of the antioxidant capacity of the sample, because the different antioxidants can act synergically to counteract the oxidative offense, and the deficiency in one particular antioxidant does not necessarily imply that the overall neutralizing capacity is impaired. Therefore, several methods have been developed to estimate the total antioxidant capacity. This considers the cumulative action of all the antioxidants present in the sample assayed, rather than just the sum of the measurable antioxidants (Ghiselli et al. 2000). Some of the most commonly used analytical methods for assessing the total antioxidant status are: total radical-trapping antioxidant parameter (Ghiselli et al. 1995), trolox equivalent antioxidant capacity (Miller et al. 1993b), oxygen radical absorbance capacity (Cao et al. 1993), the ferric reducing ability of the sample

(Benzie and Strain 1996), or the capacity of a massive dose of hypochlorous acid to oxidize the antioxidant pool (Trotti et al. 2001).

4.2.1.- Endogenous regulation of antioxidants

As a consequence of this interplay among antioxidant substances, it is not surprising that different endogenous regulatory mechanisms are influenced by an external antioxidant supply. Although further research is needed to fully understand all the regulation pathways in dairy cattle, recent studies suggest that these mechanisms might be behind some of the controversial findings related to antioxidant supplementation: The nuclear factor E2-related factor 2 (Nrf2) is a redox-sensitive transcription factor that controls the transcription of genes encoding various antioxidative and cytoprotective proteins, i.e. transcriptional regulation of GSH-Px 2 (and possibly also GSH-Px 1) is Nrf2 dependent (Lubos et al. 2010). ROS can activate Nrf2 (Nair et al. 2008), which in turn counteracts proinflammatory signalling pathways (Kim et al. 2010). In periparturient dairy cattle, in addition to the increase in ROS production, animals are typically in an inflammatory-like condition (Bertoni et al. 2008; Trevisi et al. 2010; 2012), especially in the liver. Gessner et al. (2013) showed that the transition from late pregnancy to the onset of lactation leads to a strong upregulation of Nrf2 target genes with antioxidative properties. Moreover, the unfolded protein response is activated in the liver of dairy cows in this period (Gessner et al. 2014), leading to an activation of Nrf2 via the PERK-pathway (Cullinan and Diehl 2006) and thereby increasing expression of antioxidant enzymes and antioxidant capacity.

These mechanisms might be physiologic means to prevent tissue damage induced by ROS production and inflammation. Therefore they represent important targets for assuring successful adaptation to the transition period. Furthermore, this endogenous regulation of antioxidative molecules might explain the fact that excessive antioxidant supplementation impairs antioxidant capacity (Rizzo et al. 2013), as high amounts of antioxidants might impair antioxidant capacity by suppressing Nrf2 due to lower levels of ROS leading to decreased expression of antioxidant enzymes. Therefore, further studies in this line could provide useful information for establishing a 'control of success' of antioxidant supplementation.

4.3.- Estimating the risk of oxidative stress

One problem that complicates the implementation of the oxidative status assessment onto farm applications is the lack of reference values of oxidative stress biomarkers (Celi 2011a); which prevents the identification of individual cows suffering OS. Unfortunately, there are some factors that might difficult the implementation of reference intervals or values for pro- and antioxidants: (i) some degree of ROS is essential for the maintenance of physiological processes, (ii) many factors influence the production of ROS, such as the diet (Gabai et al. 2004; Pedernera et al. 2010), environmental temperature (Bernabucci et al. 2002; Tanaka et al. 2011), milk yield (Löhrke et al. 2004; Castillo et al. 2006; Pedernera et al. 2010) or body condition at calving (Bernabucci et al. 2005); (iii) animals under identical housing and feeding conditions show a great individual variability with regard to the adaptation from pregnancy to the onset of lactation (Kessel et al. 2008), reflected also in different biomarkers of oxidative stress (Castillo et al. 2005; Abuelo et al. 2013).

However, biomarkers of other processes occurring around the time of calving, despite facing similar obstacles, have been successfully translated onto on-farm applications. Using lipid mobilization as an example, hitherto the most widely studied biomarkers of transition cow health testing; there is now plenty of epidemiological data supporting the use of blood levels of beta-hydroxybutyrate (BHB), a ketone body, and non-esterified fatty acids (NEFA) to predict the likelihood of disease events or impairment of production outcomes at the herd level (McArt et al. 2013; Ospina et al. 2013), using thresholds for these parameters that were determined from statistical associations between these analytes and subsequent health and performance (Ospina et al. 2010c; b; a).

As aforementioned, the imbalance between pro- and antioxidant is what defines OS. A great oxidative challenge is not an issue when the animal is equipped with a good antioxidative defence that is able to counteract it. On the other hand, a slight increase in ROS production could have really detrimental effects if the antioxidant capacity is overwhelmed. Therefore alongside quantification of both components of the balance must be performed to provide the real picture of the redox balance (Costantini and Verhulst 2009). In addition, studies in human medicine have highlighted that the jointly evaluation of pro- and antioxidants through an index or ratio provide a better relationship with pathology than the independent evaluation of them (Sharma et al. 1999).

This approach has also been assessed in bovine research (Abuelo et al. 2013), with the so-called ‘Oxidative Stress index’ representing the ratio between ROS and total antioxidant capacity, and indicated more accurately the oxidative status of the animals. Another recent study by Cigliano et al. (2014) also implemented this approach, considering separately indexes for lipid and protein oxidative damage and concluding that they effectively describe physiological changes associated with the onset of lactation. Therefore, it is suggested this ratio to be calculated to obtain a more objective assessment of the redox balance of the animals and, thereby their risk of suffering oxidative stress. This might facilitate the interpretation of oxidative status assessment, bringing it one step forward to its on-farm application.

5.- Benefits of antioxidant supplementation

Vitamins and certain trace minerals, such as selenium (Se), have been proven to be effective in counteracting oxidative stress and the severity of several dairy cattle diseases such as mastitis or metritis (Spears and Weiss 2008; Bouwstra et al. 2009; Sordillo and Aitken 2009), both through a direct antioxidant effect and by enhancing the immune response. The NRC nutrient requirements of dairy cattle (NRC 2001) entails a very detailed and comprehensive chapter on vitamins and a section within the minerals' chapter dedicated to trace elements. However, these requirements have been traditionally established aiming to prevent deficiency diseases and there is now evidence that supplementation slightly above these reported requirements can improve animal health status and performance, as well as the quality of the final product (Castillo et al. 2013).

However, it is worth noting that when this supplementation exceeds certain to date unknown level, it can cause harmful effects, such as the increase of odds for mastitis (Bouwstra et al. 2010b; 2010a); because the excessive supplementation with antioxidants can increase the production of ROS (Rizzo et al. 2013). OS is part of a complex system and consequently many confounding factors are likely at play, which explains why some controversial results have been found in different supplementation trials. Therefore it is important to make decisions based on the evidence provided by the literature for the implementation of antioxidant supplementation strategies in dairy farms. In this section, the impact of different supplementation strategies on the animals' health status and performance will be reviewed and discussed.

5.1.- Strategies for antioxidant supplementation

Antioxidants can be synthesized in the body, derived from the diet or administered parentally. In ruminants, some vitamins do not require external supply, i.e. vitamin K can be synthesized by the ruminal and intestinal flora and vitamin D by the ultraviolet radiation on the skin; and several natural feedstuffs are also rich in antioxidants, such as vitamin E or precursors of vitamin A (NRC 2001). However, relying exclusively on these naturally produced amounts could place the animals at risk of deficiency diseases, because of the large variability in vitamin concentrations in feeds and exposure to sunlight (NRC 2001). Besides, several dairy farms keep their animals indoors, where the exposure to sunlight and fresh forages is limited; and most natural vitamins are degraded at short term after ensilage (NRC 2001). Therefore, it is necessary to supplement these animals with vitamins and trace elements, but considering that the requirements for grazing cattle might be lower than their counterparts fed conserved forages. In addition, it is recommended to give an extra supplementation in moments of augmented demands, such as around the time of calving (NRC 2001).

Among the methods available for antioxidant supplementation, the addition of vitamins and minerals to the diet of the animals is probably the most commonly used in commercial dairy farms, especially in the form of premixes added to the total mixed ration. However, as the needs for antioxidants are increased in moments of augmented metabolism, such as the transition period, in farms with a number of animals not large enough to implement practically a specific diet for close-up dry cows; the injection of vitamins and trace minerals to these animals facilitates the supplementation without requiring to create a particular management group of cows in this period. For this purpose several products are available in the market, either for single vitamins and trace elements or a combination of these.

Vitamin E and Se, are probably the most widely antioxidants included, either alone or combined, in the diets of dairy cattle. Accordingly, most of the research has focused on the effects of these substances. Vitamin E is a potent lipid-soluble, chain-breaking antioxidant (Traber and Stevens 2011) that prevents the propagation of free radicals in membranes and in plasma lipoproteins (Traber and Atkinson 2007). On the other hand, most of the antioxidant functions of Se are attributed to the action of this trace element as a cofactor for selenoproteins -including GSH-Px-; however, a recent review has highlighted the direct role of Se in counteracting oxidative stress and regulating immunity in dairy cattle around the time of calving (Sordillo 2013).

Decreased plasma levels of vitamin E is a typical phenomenon in cows at the onset of lactation (Politis 2012). However, the reason for lower α-tocopherol -vitamin E- concentration at the end of pregnancy is not fully understood. It may be due in part the use of antioxidants for colostrum synthesis (Goff and Horst 1997), but also the inflammatory-like condition in the liver of dairy cows during the transition period leads to a decreased hepatic production of different vitamin carrier proteins (Abd Eldaim et al. 2010), resulting in lower plasma levels of vitamin E. Hence, supplementation with relatively high vitamin E levels is needed to prevent the drop in plasma α-tocopherol concentrations around parturition (Spears and Weiss 2008).

5.2.- Udder health and milk quality

It is well known that changes in the mammary gland start during the last term of pregnancy, with colostrogenesis beginning several weeks before calving (Brandon et al. 1971). These changes imply an increased production of ROS and cytokines in the udder as lactation commences, simultaneously with an increase of the risk of suffering an intramammary infection (Sordillo 2005; Baldi et al. 2008; Spears and Weiss 2008), and it is just at this time when nutrition has a major impact on udder health (Baldi et al. 2008).

5.2.1- Clinical mastitis incidence

Cows with plasma α-tocopherol concentrations at calving lower than 3.0 mg/mL were at 9.4 times greater risk of having mastitis within the first 7 days in milk than cows with higher concentrations (Weiss et al. 1997). However, it was suggested that the effect of vitamin E supplementation on mastitis depends on the microorganism causing the infection, being more effective against environmental mastitis (Allison and Laven 2000).

Vitamin E supplementation during the dry period had always been thought to have either beneficial or no detrimental effects on the animals' health status (Weiss et al. 1997; Persson Waller et al. 2007). However, a recent study in The Netherlands (Bouwstra et al. 2010b; 2010a) investigated the effects of supplementing high levels of vitamin E, and concluded that this practice could increase the odds of clinical mastitis if the animals already have high levels of this antioxidant (> 14,5 µmol/L) at dry-off. This could be explained by the fact that excessive antioxidants can result in increased ROS (Rizzo et al. 2013).

Notwithstanding, meta-analyses have followed (Zeiler et al. 2010; Politis 2012), concluding that supplementing vitamin E and Se reduced the mean relative risk of mastitis by 34%, with this effect being stronger when only Se was supplemented (-40%) than when only vitamin E was offered (-30%); and suggesting to maintain the level of vitamin E supplementation to 3000 IU/cow per day in the close-up dry period, as these controversial findings were not repeated and further research is needed before changing these recommendations (Politis 2012).

5.2.2- Somatic cell counts

High somatic cell counts (SCC) are associated with clinical or subclinical mastitis, and therefore this parameter is usually employed as an indicator of udder health. Traditionally, the levels of antioxidants, such as vitamin E and Se have been associated with improved udder health (Baldi et al. 2000; Politis et al. 2004; Nyman et al. 2008; Moeini et al. 2009). A meta-analysis revealed that supplementation with vitamin E and Se reduced on average the SCC by 24. 000 cells/mL of milk (Zeiler et al. 2010).

Studies on Se supplementation alone showed that it tended to reduce the prevalence of intramammary infections and decrease the prevalence of quarters with high somatic cell count at calving (Ceballos-Marquez et al. 2010). On another study, the authors failed to identify an association between the concentration of Se and the SCC at the level of bulk tanks; although they identified that higher bulk tank Se concentration was associated with a lower risk of being a *Staphylococcus aureus* positive herd (Ceballos-Márquez et al. 2012). Dairy cows supplemented parentally with a combination of different trace elements (Se, Cu, Zn and Mn) showed lower SCC levels (decreased incidence of subclinical mastitis) in comparison to the control cows (Machado et al. 2013); although this supplementation did not affect energy metabolites or immune and oxidative stress parameters (Bicalho et al. 2014).

However, there are also other studies that failed to identify any benefit of vitamin E supplementation on SCC or association between the levels of plasma vitamin E and SCC in milk (Ndiweni et al. 1991; Jukola et al. 1996; Erskine et al. 1997; LeBlanc et al. 2002; Wichtel et al. 2004; Sivertsen et al. 2005; Persson Waller et al. 2007). In this line, it is important to remind that supplementation with vitamin E results in different changes in the redox balance in blood, liver and milk and therefore if vitamin E status is calculated only based on blood values alone; this cannot be directly extrapolated to the whole organism.

5.2.3- Milk yield and milk composition

The effect of vitamin E supplementation on milk yield varies considerably in the literature. While there is evidence that increasing vitamin E content in the diet results in increasing the daily amount of milk yielded (Wichtel et al. 2004; Moeini et al. 2009), other authors observed no effect of vitamin E supplementation on milk yield (Ortman and Pehrson 1999; Baldi et al. 2000). However, no study reported decreased milk yield associated to this practice. A meta-analysis concluded that an increase of 1.0 kg milk per animal per day can be achieved (Zeiler et al. 2010). However, it is noteworthy that most investigations reported an increase in dry matter intake associated with this supplementation; and thus the improved energy and protein supply alone could be the direct responsible of the increased milk yield.

It has been found that milk fat depression caused by crude fibre poor diets can be attenuated by vitamin E supplementation (Charmley and Nicholson 1994; Focant et al. 1998). And this fact might be attributable to the changes found in the ruminal flora associated with oral vitamin E supplementation (Naziroğlu et al. 2002). Conversely, other studies did not find differences in milk composition -fat, protein and lactose- associated with vitamin E supplementation (Baldi et al. 2000; Politis et al. 2004; Wichtel et al. 2004).

Regarding the supplementation with trace elements, Se supplemented alone had a marginal effect on increasing milk yield (Zeiler et al. 2010), however, also controversy exists in the effect of supplementation of trace elements on milk production: Se in combination with iodine and cobalt in the form of a ruminal bolus increased milk production (Cook and Green 2010), but when supplemented parentally in combination with Zn, Mn and Cu no effect on milk yield was found (Machado et al. 2013). However, the study of Cook and Green (2010) was performed only in one farm, whereas the trial of Machado et al. (2013) was conducted on three different farms and enrolled a significantly higher sample size (1416 vs. 138). Nevertheless, although discrepancies exist in the literature as to whether supplementation with antioxidants directly increases or not milk yield, this practice has not been reported to reduce it and therefore, on light of the additional beneficial effects, it is recommended this practice to be maintained in dairy herds.

Reviewing the effects of antioxidant supplementation on the quality of milk for human consumption is beyond the scope of this article, and readers are pointed to the review article by Castillo et al. (2013) for this purpose.

5.3.- Uterine health and reproductive performance

Postpartum uterine diseases, such as retained foetal membranes (RFM), endometritis or metritis have a major impact on dairy farms economy (Laven and Peters 1996), as they are associated with poor fertility and decreased milk production, on top of the treatment costs. The aetiology of these diseases is not fully understood, but as retained placentas showed impaired antioxidant activity (Kankofer 2000) and increased oxidative damage (Kankofer 2001); it has been hypothesized that oxidative stress plays a key role in the improper release of the afterbirth. However, the effectiveness of vitamin E supplementation in reducing RFM incidence is controversial, with half of the studies demonstrated positive effects of vitamin E, whereas the other half showed no benefit (Politis 2012). Early studies showed that the incidence of RFM was reduced by supplementation with Se regardless of the supplementation with vitamin E (Julien et al. 1976). Afterwards, it has been suggested that the interactions between vitamin E and selenium are responsible for the effectiveness of vitamin E (Allison and Laven 2000).

However, higher plasma concentrations of α-tocopherol were associated with a decreased risk of RFM (LeBlanc et al. 2002). In addition, a meta-analysis tried to account for the effect of vitamin E on several trials (Bourne et al. 2007), finding that vitamin E was associated with a decrease in the incidence of RFM and also concluded that the synthetic form of vitamin E was more effective than the natural one; although the authors were unable to explain this finding. It is noteworthy that the benefits of this supplementation may depend on whether or not cattle have adequate plasma vitamin E before supplementation (Schingoethe et al. 1982). This further highlights the necessity of tools that can practically assess the necessity of antioxidant supplementation on cattle.

A recent well-designed study (Bicalho et al. 2014) has shown that cows affected by RFM had also reduced serum concentrations of Ca, Mg, Mo and Zn, those affected by metritis lower concentrations of Ca, Mo, P, Se, and Zn and the ones that showed endometritis had significantly lower levels of Ca, Cu, Mo, and Zn compared to their non-affected counterparts. Cows injected with a trace element supplement (Se, Zn, Cu and Mn) showed a lower risk of developing endometritis and metritis (Machado et al. 2013) and, in addition this practice decreased the presence of *Fusobacterium necrophorum* and *Trueperella pyogenes* in the uterus at 35 days after calving (Machado et al. 2012).

However, further research is needed to fully understand the mechanisms of action, since this injectable supplementation did not seem to affect the serum concentration of NEFA or BHB, the activity of antioxidant enzymes (GSH-Px and SOD), the production of pro-inflammatory cytokines or the concentration of haptoglobin -a positive acute phase protein- (Bicalho et al. 2014).

Antioxidant supplementation can potentially improve cows' fertility by reducing the incidence of these disorders of the uterine environment, as they have a direct effect on pregnancy success after breeding (Lopez-Gatius et al. 2006). On the other hand, despite ROS playing several physiologically essential roles in reproduction (Rizzo et al. 2012), when produced in excess they are also related to ovarian diseases and reduced reproductive performance (Rizzo et al. 2009; 2012), and an enhanced antioxidant potential could counteract these harmful effects. However, the results of different field trials have been inconsistent. Supplementation with vitamin E and Se 21d before expected calving increased pregnancy rates (Arechiga et al. 1994), but when supplemented 30d after calving, it did not improve first service conception rate (Arechiga et al. 1998). In the study by Campbell and Miller (1998), supplementing cows with vitamin E at a 1000IU/day rate during the last 42d prepartum reduced the interval from calving to first oestrus. However, vitamin E and Se supplemented before and after calving did not improve reproductive performance in other studies (Paula-Lopes et al. 2003; Bourne et al. 2008).

The role of minerals in reproduction has been also extensively investigated. Conception rates and days to first service were improved by supplementation with Cu, Zn, Mn and Se, and fertility was further improved when part of these elements were given in organic form instead of inorganically (Ballantine et al.

2002; DeFrain et al. 2009). Heifers that received trace minerals subcutaneously 17d before embryo transfer showed a higher conception rate (Sales et al. 2011). However results are not consistent either for the effect of trace elements in reproductive performance, as the multi-farm study of Machado et al. (2013), involving more than 1400 cows, failed to identify any improvement in reproductive parameters.

5.4.- Incidence of other production diseases

The displacements of the abomasum (DAs) are one of the production diseases frequently found in postparturient dairy cattle with a multifactorial origin (Van Winden and Kuiper 2003), requiring in most cases surgical correction. The incidence of DAs causes a major impact in the dairy industry (Geishauser et al. 2000) and the economics losses include both the treatment of culling costs and the reduced milk production (Doll et al. 2009). Cows with DA have 40% lower serum concentration of antioxidants than their non-affected counterparts (Mudron et al. 1997; Hasanpour et al. 2011; Mamak et al. 2013), with lower levels of vitamin E preceding DAs' onset (Qu et al. 2013); further suggesting a direct implication of oxidative stress in the pathogenesis of this health disorder.

However, in one field trial, vitamin E supplementation did not decrease the incidence of left DA (LeBlanc et al. 2005) in comparison to a placebo group. Notwithstanding, the plasma levels of α-tocopherol after the first week following supplementation were, in both supplemented and placebo groups, below the recommended levels of 3 µg/mL (Weiss et al. 1997) for periparturient cattle. Therefore, further research is needed to fully understand the implications of

ROS production in the pathogenesis of DAs and the potential benefits of antioxidant therapy.

6.- Conclusions and future considerations

The transition from late gestation to the onset of lactation imposes a high and quick metabolic adaptation for dairy cattle, predisposing them to health disorders that affect their overall performance and impact the economy of the farms. OS plays a key role in the pathogenesis of these diseases and has been identified as a link between nutrient metabolism and inflammation during this period (Sordillo and Mavangira 2014). Thus, controlling this condition through appropriate antioxidant supplementation could potentially ameliorate the animals' health status and performance.

However, hitherto antioxidant therapies have not consistently achieved this goal. Yet, to the best of the authors' knowledge, no study has evaluated adapting supplementation strategies to the initial antioxidant capacity of the animals. As an excessive amount of antioxidants has the potential of increasing the production of ROS, and has been associated with lack of supplementation effectiveness or even increased odds for health disorders; it is necessary to provide tools that can be used on farms to stablish nutritional strategies for preventing diseases early enough in the far-off dry period. Thus, standardization of oxidative status assessment in dairy cattle is vital, as a first step for establishing critical thresholds that could aid in defining appropriate protective nutritional strategies on the basis of antioxidant supplementation.

Acknowledgements

Authors wish to thank the Galician Government (Xunta de Galicia) for funding their research on oxidative stress in dairy cattle under grants 10MRU261004PR and CN2012/327. The funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. A. Abuelo is holder of an FPU fellowship (Ref. AP2010-0013) from the Spanish Ministry of Education, Culture and Sports.

Conflict of interest statement

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Figure captions

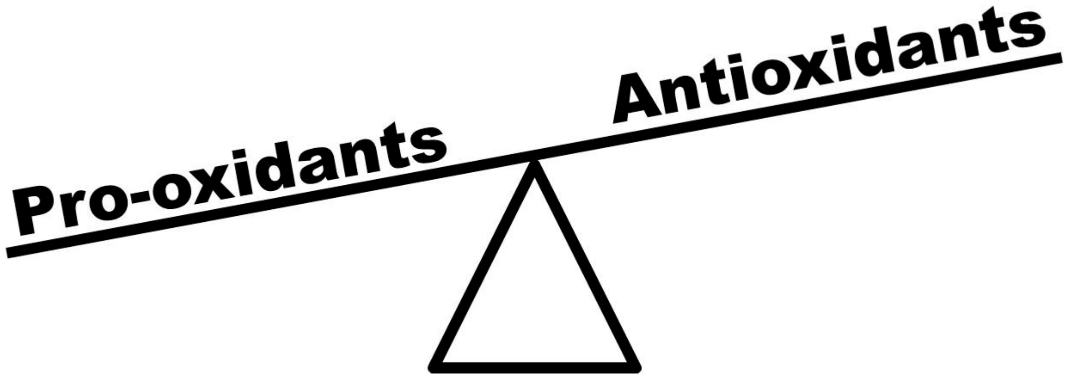

Figure 1.- Schematic representation of the redox balance within an organism.

http://dx.doi.org/10.1111/jpn.12273

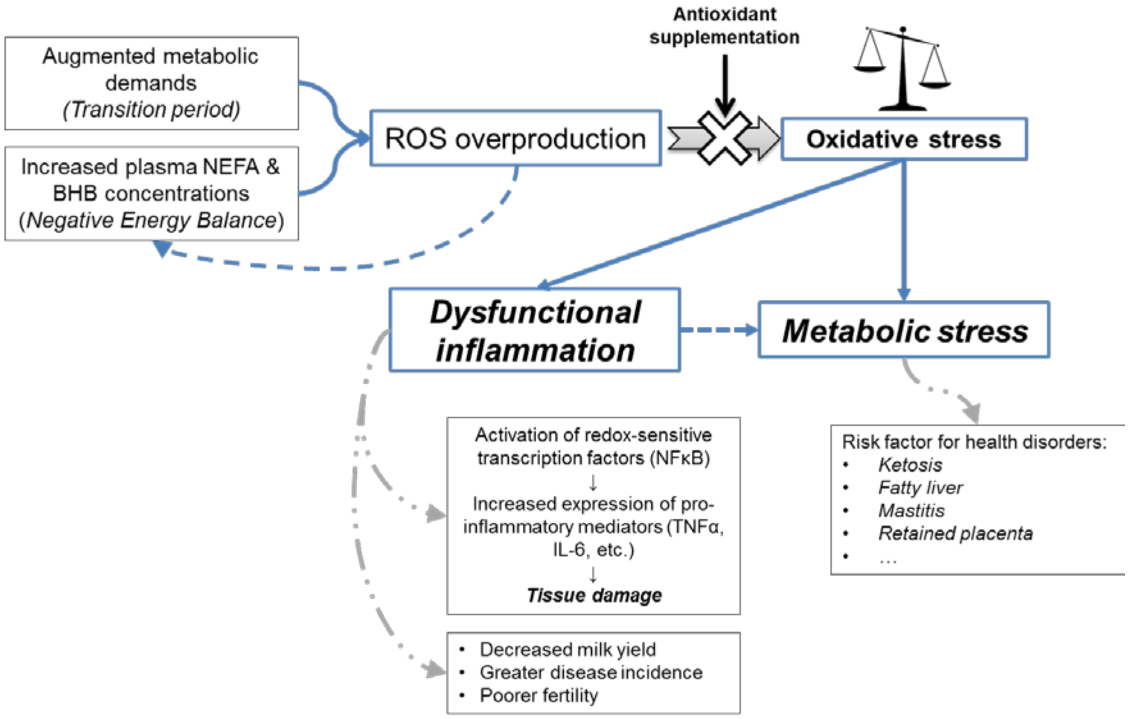

Figure 2.- Schematic illustration of the interplay between antioxidant supplementation, metabolic stress, dysfunctional inflammation and presented health disorders.

NEFA = Non esterified fatty acids; BHB = β-hydroxybutyrate; ROS = Reactive oxygen species; NFκB = nuclear factor kappa B; TNFα = tumor necrosis factor alpha; IL-6 = Interleukin 6.