Redox status and oxidative stress in bovine

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SUMMARY

In the last few years, we have seen a marked increase in the number of articles emphasizing the role of oxidative stress in the pathogenesis of multiple cattle diseases and processes. Since the term oxidative stress (OS), defined as the imbalance between prooxidants and antioxidants, was coined, the physiological and pathological roles of both have also been described. This has shown that understanding the relationship between the two components, known as redox status, is a very useful tool in establishing the health and disease status of cattle. OS has been related to production diseases, metabolic diseases - such as ketosis, fatty liver, and even hypocalcaemia - and reproductive diseases. These include both maternal diseases - like those connected with numerous pathologies such as placental retention, udder oedema, or mastitis - and foetal growth. Finally, the term metabolic stress has been established. It relates OS itself to lipomobilization and immune system dysfunction, similar to the so-called metabolic syndrome in humans. Metabolic stress describes the catabolic response to the alteration of physiological homeostasis and is characterized by excessive lipid mobilization, immune and inflammatory dysfunction, and OS.

KEY WORDS

ROS, redox status, internal metabolism, cattle.

INTRODUCTION

The interest in oxidative stress (OS) and its role in the genesis of many diseases has not stopped growing since it was coined by Professor Sies in 1985[1]. It is defined as a situation of "imbalance between the number of pro-oxidants and antioxidants, in favour of the former". This definition was expanded by Sies and Jones in 2007[2], as they pointed out the important role of oxidants (ROS, Reactive oxygen species) in biological activity, particularly, their role as signalling molecules and their physiological action at low concentrations, in a mode of action known as "redox signalling"[3]. It is important to mark that redox status could be defined as the balance between oxidants (or pro-oxidants) and antioxidants, and the OS is the imbalance between them. In other words, redox biology refers to low levels of ROS that activate signalling pathways to initiate biological processes while oxidative stress denotes high levels of ROS that incur damage to DNA, protein, or lipids.[4]

The importance of OS lies in pointing out free radicals, or prooxidants (ROS) as molecules that play very important roles, from the physiological point of view, in the internal balance of cattle[5]. However, it is important to remark that the missions described so far are not all the missions in which they participate, as new missions in the individual are discovered and described every day.

To give a numerical figure about the importance of OS and its presence in clinical activity, 145,000 entries appear in the thematic search engine Google Scholar after requesting information on oxidative stress in the last year, and of the total number of entries, 17,000 refer exclusively to cattle. If we were to focus on the search in the year 1985, when Professor Sies[1] coined the term and made it popular, we would find 355 entries referring to the concept in relation to cattle. Therefore, it seems logical to conclude that oxidative stress was, is, and will always be a very important aspect to consider by clinical veterinarians, and especially by researchers. It is also important to highlight the great interest oxidative stress has generated from the very moment the concept arose. However, and due to the analytical limitations when determining free radicals, the first researchers were forced to consider OS from a single perspective, from the antioxidant point of view and in a partial way, since the investigations only evaluated the role of a vitamin or mineral individually. As for the course of already established diseases, their genesis was attributed to a deficiency or excesses of vitamins or minerals[5]. The measurement of free radicals was left to physicists and chemists because of their complexity. However, they were not recent compounds. Indeed, Gomberg first described them in 1900[6] as the result of the decomposition of Hexa-phenolate into two triphenylmethyl radicals.

To put it simply, the concept of OS could be compared to a swing which represents a state of equilibrium between two forces. Thus, we could deduce that the ideal situation is to maintain an equilibrium between ROS and the system that neutralizes them, the antioxidants. This way, we should not be concerned about an increase in ROS production, a frequent occurrence in cattle. It should not be forgotten that these radicals arise from the production of energy by the utilization of oxygen, as long as the antioxidant system is also modified (increased) proportionally, thus maintaining the situation of equilibrium between the two.

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Returning to the example of the swing, if there were an excess of ROS, we would have OS, and if there were an excess of antioxidants, we would have a deficit of oxidants. That is, a deficit in the utilization of oxygen which would be as negative as the oxidative stress itself, since, in addition to the fact that nobody wants to live with an oxygen deficit, its low presence would mean a decrease in the productive capacity of the individual. Balance must be our virtue and our target, and we must avoid both extremes as far as possible. What is also true is that a certain degree of OS not always harms the organism, because it helps to maintain the internal environment "in tension or alert". That is, ready to respond to special circumstances that may occur, such as a change in demands due to a change in the bovine's productive state[7].

The components of this combination are free radicals (ROS) and antioxidants. Thus, free radicals, chemically speaking, are any molecule or atom that has one or more unpaired electrons in its last shell. This gives it a double particularity: high instability and, as a consequence, high reactivity. The result is the generation of more radicals, increasingly unstable and more reactive, that are formed upon contact with other molecules which may be previously unstable or stable[5]. From a chemical point of view, the reactions can be derived from a process of oxidation (electron transfer) or reduction (electron capture), whereby the molecule that captures the electron becomes reduced, and the molecule that loses it will be oxidized[6].

Synthesis, physiological functions and classification of ROS and antioxidants

Synthesis

The main source of ROS production comes from oxygen metabolism. Thus, cells metabolize a 95% of the oxygen by tetravalent reduction, binding to protons to produce water, which is not a problem for the organism. However, the remaining 5% is monovalently reduced. That is, one oxygen molecule plus four electrons and four protons from two water molecules and three highly toxic intermediates, two free radicals (superoxide and hydroxyl anion) and a peroxyl radical[8,9]. Figure 1 shows a schematic representation of the double pathway of oxygen metabolism.

Physiological functions

A major aspect that we should never forget is that free radicals fulfil very important physiological missions in bovines, as mentioned above. Thus, we can point out their important role in the immune system of animals, through the well-known process of respiratory burst of phagocytes. This action could be defined as that situation in which some cells can produce and release reactive oxygen species, such as superoxide radicals and hydrogen peroxide[9]. It is characterized by a very violent increase in both oxygen demand and energy consumption at the cellular level. This mechanism is frequently used by cells of the immune system to produce compounds with microbicidal capacity, not only against bacteria but also against parasitic infections. Some of those compounds are hydrogen peroxide, hypochlorous acid, nitric oxide and peroxynitrite[10]. In addition, free radicals or their derivatives participate in the regulation of the vascular tone, the oxygen pressure perception, and the regulation of functions that are controlled by the oxygen concentration. Moreover, they potentiate the intracellular signal transduction of various membrane receptors, including the lymphocyte antigen receptor, and chemical reactions that ensure the maintenance of the redox system[6]. The hydroxylation of the amino acids lysine and proline to hydroxylysine and hydroxyproline, necessary for collagen biosynthesis, requires the participation of the hydroxyl radical. Another very inter-

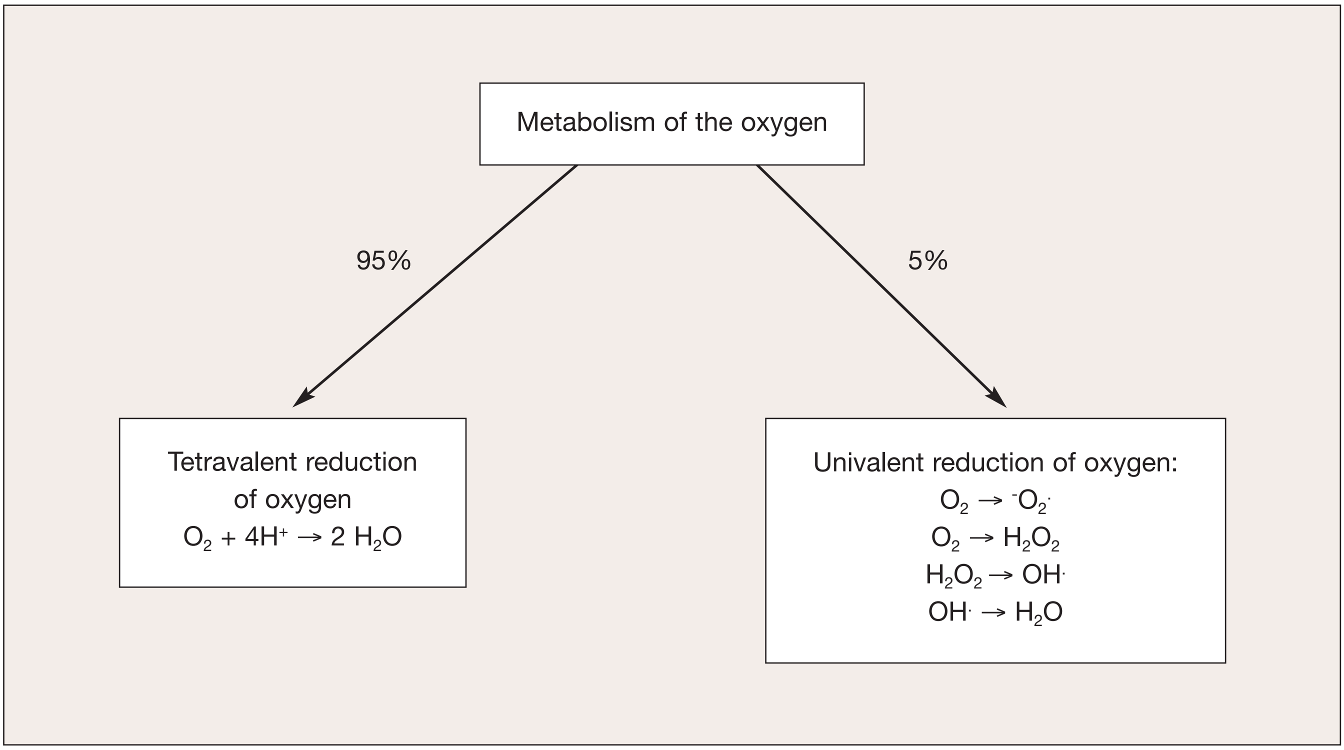

Figure 1 - Pathway of Metabolism of the oxygen (taken from [5]).

esting aspect of free radicals is that they can be considered as neurotransmitters because, besides facilitating the transmission of nervous information between cells, they favour the synthesis of glutamate. They also contribute to regulating the luteal phase in the cow, as they participate in the synthesis of progesterone. Finally, they are involved in the synthesis of prostaglandins, cholesterol, and steroid hormones[11,12].
The negative aspect of free radicals could be related to the formation of ROS, including the formation of unstable and highly reactive nitrogen species which, once formed, contact the phospholipids of cell membranes and originate peroxidation of membrane lipids. This occurs when a free radical is attached to a carbon of the alkyl chain (CH3-(CH2)n-) of a fatty acid and initiates the process known as lipid peroxidation[13]. The importance of all this lies in the fact that the fatty acid chains, particularly the polyunsaturated ones (PUFA), are fragmented so that the phospholipid structures of the membranes are disorganized and destroyed. They also react with the thiol groups of the sulfurized amino acids producing the less famous, but not less important, nitrosative stress and inducing changes in the conformation of the proteins. Therefore, as regards their functionality, the most affected structures are the membrane proteins (ion channels, receptors, etc). Other affected structures are the proteins that form part of the contractile machinery, particularly the Na+/K+ATPases and the ATPases linked to the proteins. Finally, free radicals are also capable of reacting with the purine and pyrimidine bases in DNA as they react with the adenosine, the guanidine and, especially, the cytosine of the DNA, leading to the appearance of alterations in the transcription of information and inducing the appearance of mutations and even favouring carcinogenesis[5].

Classification ROS and antioxidants

Among the many free radicals described, Table 1 shows the best known and their most frequent origin.
In contrast to free radicals, we have the defensive system. That is, the antioxidants, which can have an endogenous or exogenous origin. Although they were formerly classified as primary, secondary or tertiary, today it is more practical to subdivide them into enzymatic and non-enzymatic. As in the previous case, Table 2 shows the best-known ones, although we would like to point out that there are many more.
At this point, we would like to point out two things. One, although known to the reader, is that many antioxidants can be converted into free radicals. A typical example is iron, which, besides being part of transferrin, is a potent catalyst in the synthesis of the hydroxyl radical. And the same example could be applied in the case of copper and ceruloplasmin. Therefore, we should not abuse the application of antioxidants. The other nuance is the fact that antioxidants, both the ones that act in a hydrophilic environment - inside the cell - and those that act in a lipophilic environment - in the cell membranes - try to repair the potentially repairable damages and eliminate those that cannot be repaired[14].

Table 1 - Main ROS in the body.

Types of ROS	Formation
Superoxide anion ($-O\bullet_2$)	Formed in autoxidation reactions (flavoproteins, redox cycle).
Hydroxyl radical ($\bullet OH$)	It is the species with the shortest half-life and is the most reactive ROS species. It interacts with the nitrogenous bases of nucleic acids, altering the genetic information of cells. Stimulates lipid peroxidation, affecting the phospholipids of cell membranes.
Peroxyl radical ($ROO\bullet$)	Formed from organic hydroperoxides or ROOH due to loss of H +.
Hydrogen peroxide (H_2O_2)	Formed from the dismutation of $-O\bullet_2$ or it can come directly from O_2.
Hypochlorous acid (HOCl)	Produced by the action of the respiratory burst of defensive cells.
Nitric oxide (NO)	Produced by the union of oxygen with nitrogen, inducing lipoperoxidation.
Ions Fe^{+++} and Cu^{++}	They act as catalysts in the formation of hydroxyl radicals.
Singlet oxygen (O_2)	It is simple molecular oxygen, the first excited state. It is formed by the activation of O_2 (sunlight, radiation).

Table 2 - Main antioxidants in the body.

Antioxidants	Location
Superoxide dismutase (SOD)	Occurs primarily in the alveolar epithelium and the endothelium.
Catalase (CAT)	Primarily intracellular with elevated activity in the liver and kidneys.
Glutathione (GSH)	Glutathione peroxidase and glutathione reductase responsible for regenerating GSH from GSSG.
Ceruloplasmin	Serum lipid peroxidation inhibitor.
Antiproteasas	Specifically, inhibit the action of proteases and the respiratory burst of neutrophils.
Estrogenic metabolites	Protect against lipoperoxidation.
Tocopherols	Fat-soluble antioxidants protect the integrity of cell membranes.
Ascorbic acid	Similar in action to CAT, although not as effective.
Carotenes	Act in hydrophobic compartments (e.g. Lycopene).
Uric acid	Antioxidant at physiological concentrations.
NADPH	Contributes to the regeneration of GSH and CAT.

The role of OS in daily clinical practice in cattle

OS and mastitis and dairy production

Let us have a look at some examples of the role of OS in daily clinical practice in cattle. First of all, we will look into the relationship between OS and mastitis. When inflammation of the udder occurs as a result of the action of phagocytic cells, cytotoxic radicals are released, as well as proinflammatory cytokines that damage the secretory epithelium of the gland. The presence of high numbers of neutrophils, macrophages, lymphocytes, and eosinophils in milk, together with desquamation of epithelial cells, is nothing more than the result of the defensive response of the mammary tissue[11,15,16].

In addition, it turns out that macrophages and epithelial cells produce high amounts of nitric oxide (NO), a mediator of inflammation, further activated by the action of released cytokines. NO acts as the first antimicrobial defensive barrier thanks to the action of peroxynitrite, a highly reactive nitrogenous metabolite derived from the oxidation of nitric oxide[17]. However, if its production is excessive, peroxynitrite can affect the lipids of cell membranes (lipid peroxidation), depleting the levels of other defensive antioxidant molecules and even destroying the cell membranes themselves through contact with their phospholipids[15,18].

Because of this relationship, some studies propose the measurement of NO levels in milk as a marker for situations of subclinical mastitis[18]. Thus, milk from cows affected by subclinical mastitis contains lower levels of antioxidants than milk from healthy cows. What is more, the latest studies carried out in cattle also indicate higher levels of reactive oxidants, with harmful effects[16,19].

Concerning OS and milk production, although it is not a pathology per se, it is worth noting the link between the two. It is evident that, in the postpartum period, the demands of milk production are even greater than those of gestation itself[13]. This induces a maximum energy requirement in the animal that makes it enter a negative energy balance which forces catabolic pathways in pursuit of energy. In turn, this process will end up generating a high number of free radicals[20].

The productive level reached will depend indirectly on the oxidative balance of the animal during this phase. Obviously, the higher the milk production, the more oxidants will be produced by the metabolization of oxygen.

OS and metabolic disturbance

Another process of particular interest would be the evaluation of the relationship between OS and dysmetabolism, such as ketosis, fatty liver, and even hypocalcaemia. The relationship among some of them, although intuited, is not yet defined. However, no one doubts that the action of free radicals on the macromolecules involved in metabolic pathways can be as harmful as the action on cellular elements.

Free radicals are present in a multitude of metabolic pathways, either anabolic or catabolic. The mere fact of altering a protein involved in any of these pathways can alter its conformation and therefore its function, interrupting the chain of biochemical reactions[5].

Both antioxidant systems and reactions catalysed by steroid enzymes require reductants provided by NADPH. When many of these equivalents are consumed (because there is a situation of oxidative stress), the levels of NADPH, essential for a multitude of vital biochemical pathways, decrease[15]. One of those NADPH levels refers to glucose metabolism and becomes particularly relevant when glucose requirements are very high as it diverts this molecule from its normal pathway.

In the cow, endocrine-metabolic changes associated with the transition period can lead to fatty infiltration of the liver subsequent to lipid mobilization[11], which damages cellular structures and impairs their function. Moreover, this situation is reinforced by an increased insulin resistance - a physiological phenomenon in the postpartum period of the cow - which is generated to favour the flow of glucose to the mammary gland[21].

Interestingly, cows with a BCS greater than 3, when forced to mobilize fat, will suffer greater oxidative stress than those animals that are leaner[20]. Bernabucci et al., in 2005[22] stated that cows with greater BCS loss are more sensitive to oxidative stress. They also reported a positive association between oxidative status with NEFA and BHB as indicators of lipomobilization and ketogenesis.

It is known that susceptibility to post-partum oxidative stress will be conditioned by the animal's body condition at calving, its milk production, and the diet it receives, as we have already pointed out[23,24]. Feeding cows high amounts of cereals (rich in starch) at the beginning of lactation, without previous adaptation, can cause alterations in oxidative phosphorylation at the rumen level and cause oxidative stress[25].

The relationship between free radical generation and hypocalcaemia - due to deficiencies in vitamin D, responsible for maintaining stable calcium levels - has also been demonstrated. In this case, high levels of ROS override the activity of the enzyme cytochrome P-450. This enzyme is responsible for stimulating 1,25-dihydroxycholecalciferol (an active form of vitamin D)[5].

In a recent article, Kweh et al., in 2021[26], demonstrated that the relationship between vitamin D and control of ROS is positive for the immune system. They also demonstrated that dependence of the effects of 25(OH)D3 (calcitriol) on antioxidant responses may be explained in part by the transcription factor NFE2L2(Nuclear Factor, Erythroid 2 Like 2), a key factor in the activation of cellular antioxidant defences.

Moreover, the fact that calcitriol is a regulator of innate and adaptive immunity in cattle through its connection with oxidative esters has made the role of vitamin D on the immune system be highlighted by different authors[27].

Bacha, for instance, in 2020[28] points out that in cattle, 1.25 (OH) 2D3 strongly increases the production of nitric oxide and antimicrobial -defensin peptides, which are toxic to bacteria. Given the close relationship between vitamin D and immune status, we should not focus on whether the excess of ROS decreases the vitamin D synthesis or not, but rather on its relationship with redox status. This relationship favours the antioxidant synthesis at the cellular level in monocytes by increasing the thioredoxin and metallothionein systems[26], where biomarkers of oxidative stress may be key outcomes to assess the effects of vitamin D treatments in cattle.

OS and reproductive disorders

As a last example, we could address the relationship between OS and reproductive alterations. In bovine medicine, and specifically in the dairy cow, oxidative stress has been connected with numerous pathologies such as placental retention, udder oedema or mastitis, as we have already pointed out. From a biological dimension, inflammation, which involves the release of chemokines and cytokines; dilation of blood vessels; and infiltration of immune cells, is the first-line immune response of an organism confronted with microbial infection or tissue injury. Since the female genital tract is physiologically exposed to various processes such as ovulation and bacterial infection after parturition or insemination, it is possible to conclude that inflammation is also part of the physiology of the reproductive tract [29].

Although the physiological level of ROS plays an important role in reproductive processes, including follicular development; oocyte maturation; luteal regression; and fertilization (Shkolnik et al. 2011), a consistent accumulation of ROS, resulting from OS, is considered one of the major stress inducers that significantly contributes to reproductive cell failure[30].

In the corpus luteum, the metabolism of steroidogenic cells and mononuclear phagocytes produces oxidants. The progressive increase in levels of oxidants acts as a regulator so that, when a certain level of oxidants is reached, the process of luteolysis and the onset of oestrus are triggered. In this process, SOD and catalase emerge as the antioxidants in charge of neutralizing the harmful effects while entering the regression phase. At this time, the decrease in blood flow to the ovary can per se be the cause of oxidative stress since the lack of oxygen will generate energy through alternative pathways. However, if the animal has sufficient antioxidant reserves, this process will hardly affect its physiology [31]. It is important to stress that the control of physiological concentrations of luteal ROS by antioxidant enzymes is a key element for CL progesterone production, whereas the uncontrolled ROS generation, due to imbalance between ROS and the antioxidant systems, is detrimental to the demise of the CL at the end of the non-fertile reproductive cycle[32]. Reactive oxygen species have been implicated in the regulation of the luteal function, including luteo-protective and luteolytic roles[33]. Leukocytes, especially eosinophils, macrophages, and T lymphocytes, are recruited into the CL within the first 5 minutes after a PGF2 injection. As early as 30 minutes later, the expression of endothelial nitric oxide synthase is stimulated, accompanied by an increase in luteal blood flow and IL8 expression[29].

When ROM levels are elevated (i.e., by increased PGF2), luteal cycles are altered by several mechanisms[31]:

- The action of the enzyme cytochrome P-450, responsible for transporting cholesterol into the mitochondria, is inhibited. Consequently, progesterone synthesis is inhibited as well.
- ROMs are capable of blocking luteinizing hormone receptors, favouring premature luteolysis.
- They also alter the integrity of the plasma membrane of luteal cells by affecting the long-chain fatty acids of their membrane, thus impairing their function.

Since the local ROS concentrations are controlled by antioxidant enzymes, it is possible that these enzymes are involved in regulating the luteolytic action of PGF (Vu and Acosta, 2014).

1. But oxidants are also generated during embryonic metabolism. Their excess is associated with the appearance of embryonic death.

Under normal conditions, embryos have antioxidant defence mechanisms, both internal (SOD and glutathione) and external (transferrin and ascorbic acid present in the oviduct). When a state of oxidative stress is established, embryo preimplantation is damaged since oxidants affect the enzymatic function, as well as the mitochondrial structures, leading to an increase in lipoperoxidation and DNA fragmentation[31].

At the level of foetal growth, the presence of OS also conditions the viability of gestation through different mechanisms, depending on the origin of the alteration. Thus, Castillo et al., in 2001[5] point out that, if the initial process is due to inflammation, the activation of the inflammatory response or the immune system will cause an increase in cytokine levels. In turn, this will cause an increase in the secretion of other molecules that are harmful for the embryonic development such as PGF2 and nitric oxide. In case of bacterial infections, the phagocytic activity of neutrophils and their respiratory burst will also release oxidants[31]. If the process is due to nutritional imbalances, a reduction in the bioavailability of tetrahydrobiopterin (BH4) - an essential cofactor for the synthesis of nitric oxide at the endothelial level and, at the same time, a potent antioxidant - may occur[5]. Furthermore, an increased level of ROS affects the reproductive potential negatively and is associated with poor oocyte quality, suboptimal embryonic development, and decreased female fertility[30]. In addition, it could promote the intrauterine growth retardation of the embryo and the foetus syndrome because the corpus luteum is the main producer of progesterone in early pregnancy. The insufficient activity of the ovary, manifested in hypoprogesteronemia, does not provide an optimal secretory response of the uterine glands nor enough embryo nutrition. However, it contributes to the aggressive reaction of peripheral mononuclear cells against the tissues of the growing placenta and the embryo itself[34]. Under normal conditions, NO regulates the action of insulin at the uterine level, favouring the glucose uptake and metabolic activity at this level. However, a reduced NO synthesis results in the development of a greater resistance to the insulin action. In turn, this leads to what is known as intrauterine growth restriction, which can bring about a premature delivery and/or foetal death[34].

Finally, we must also mention the existence of an association of three very common metabolic states in dairy cows. These are the state of lipid mobilization, alterations of the immune system, and OS. The term used to refer to the coexistence of these three states is *metabolic stress*. Metabolic stress, which is similar to the so-called metabolic syndrome in humans, describes the catabolic response to this alteration of physiological homeostasis and is characterized by excessive lipid mobilization, immune and inflammatory dysfunction, and OS (Figure 2). These three processes are intrinsically related and result in immune and metabolic disorders that are associated with an increased risk of metabolic and infectious diseases during this period [20].

The classic triad, initially described by Sordillo and Aitken in 2009[8], shows that the presence of excess ROS has a double effect as, besides favouring the appearance of inflammation by activation of the NF-kb factor (Nuclear factor kappa-light-chain-enhancer of activated B cells), which conditions the appearance of an inadequate immune response, it also favours lipolysis. Furthermore, the inflammatory response of the individual will also have a double effect derived from the release into the organism of inflammatory cytokines that will favour the overproduction of ROS and the inhibition of dry matter intake, favouring lipid mobilization[13]. Finally, as with the presence of ROS, lipid mobilization will promote the appearance of inflammation by activation of the NF-kb factor[35]. Therefore, we could point out that the presence of a single one of the three states mentioned above, strongly favours, although it does not guarantee, the simultaneous presence of one or the other two mentioned above.

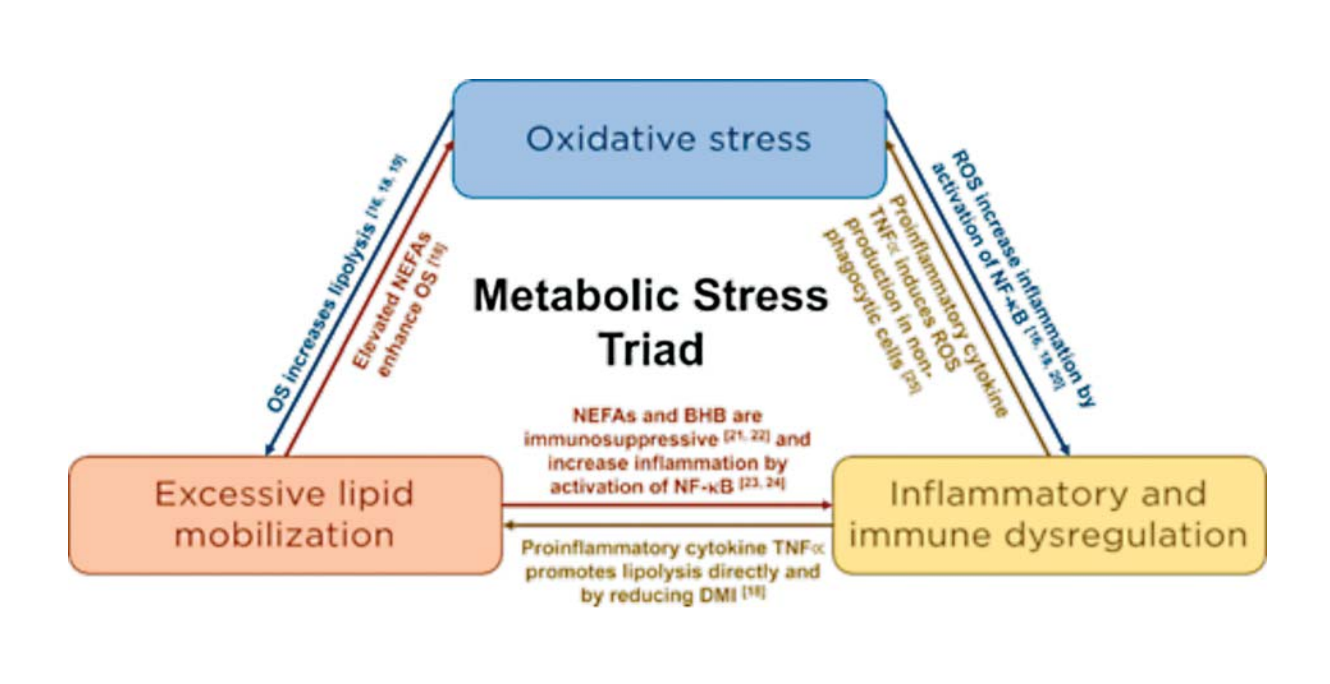

Figure 2 - Relationship between oxidative stress, lipid mobilization, and inflammatory and immune dysregulation (Obtained from [20]).

CONCLUSION

To conclude, we could point out that the presence of free radicals should not be considered, a priori, a negative situation for the organism, since they fulfil strategic missions in the animal physiology. The antioxidant defence should be able to neutralize any change registered in the ROS. Therefore, it is very important to establish the relationship between the two components, the so-called redox status, before indiscriminately applying antioxidant solutions without control, because we must not forget that some antioxidants, such as copper or iron, can become prooxidants. We should also keep in mind that a certain degree of OS can contribute to keep the internal environment on alert, thus contributing to avoid or minimize the development of certain pathologies. Finally, oxidative stress is not in itself a disease, but a state of imbalance that participates in the genesis of many diseases.

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