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Kirschvink, Nathalie; Moffarts, Brieuc de; Lekeux, Pierre

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Review

The oxidant/antioxidant equilibrium in horses

Nathalie Kirschvink [a,*], Brieuc de Moffarts [b,1], Pierre Lekeux [b]

[a] *Animal Physiology, Department for Veterinary Medicine, Faculty of Sciences, University of Namur, Belgium*
[b] *Department for Functional Sciences, Faculty of Veterinary Medicine, University of Liège, Belgium*

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Abstract

Since "free radical research" started in 1954, understanding the role of oxidants and antioxidants in physiological and pathological conditions has increased continuously. Oxidants are essentially generated by metabolic enzymes, inflammatory cells and mitochondrial electron leakage; they are indispensable for the cellular redox regulation and may, under certain conditions, have a pro-inflammatory stimulatory role. Endogenous and exogenous antioxidants counterbalance the oxidative processes and so maintain the oxidant/antioxidant equilibrium. Excessive oxidant generation or antioxidant insufficiency can lead to oxidative stress. The aims of this review are: (1) to provide an insight into the concept of the oxidant/antioxidant equilibrium by briefly introducing the oxidant and the antioxidant systems; (2) to describe how the oxidant/antioxidant equilibrium or oxidative stress can be evaluated in horses, and (3) to summarise current knowledge about oxidative stress in equine medicine and equine exercise physiology.

Keywords: Equine; Oxidants; Oxidative stress; Pathology; Exercise; Antioxidant therapy

Introduction

"Free radical research" started in 1954 when Gerschman and collaborators (Gerschman et al., 1954) published the free radical theory of oxygen and when, two years later, Harman published the free radical theory of aging (Harman, 1956). The field gained further attention in 1969 when McCord and Fridovich discovered the enzyme superoxide dismutase (SOD; McCord and Fridovich, 1969), but the most important literature has been published during the last two decades.

Our understanding of the role of oxidants and antioxidants in physiological and pathological conditions is continuously increasing and some oxidant-associated or oxidant-mediated processes are now considered as future therapeutic targets (Rahman et al., 2006). Interestingly, an important part of animal research in this field has been performed in horses, in particular with regard to exercise physiology.

The aim of this review is to provide insight into the concept of the oxidant/antioxidant equilibrium by introducing the oxidant and antioxidant systems, describing how the oxidant/antioxidant equilibrium or oxidative stress might be evaluated in the equine species and by presenting current knowledge about oxidative stress in equine medicine.

The oxidant system

Definitions

Reactive oxygen species (ROS) or *oxidants* can be defined as oxygen-containing molecules that are more reactive than the oxygen molecule present in air (Noguchi and Niki, 1999). ROS include free radicals as well as reactive compounds without unpaired electrons in their outer orbit. Such non-radical oxidants include peroxynitrite ($ONOO^-$), hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl).

Free radicals are molecules or molecular fragments containing one or more unpaired electrons in atomic or

* Corresponding author. Tel.: +32 81 72 43 79; fax: +32 81 72 44 20.
E-mail address: nathalie.kirschvink@fundp.ac.be (N. Kirschvink).
[1] Present address: Pavesco AG, Basel, Switzerland.

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molecular orbits, which considerably increase their reactivity (Halliwell and Gutteridge, 1999). The radical group includes species such as nitric oxide ($NO^{\bullet}$), superoxide ($O_2^{\bullet-}$) and hydroxyl radical ($HO^{\bullet}$).

Antioxidants are included in the defence systems against oxidants, which imply (1) systems that prevent ROS generation, (2) antioxidant systems that inactivate oxidants, and (3) systems able to limit the deleterious effects of oxidants by allowing repair of oxidative damage (Cheeseman and Slater, 1993). The antioxidant defence system will be further detailed in Section "The antioxidant defence system".

Oxidative stress has been defined as a disturbance of the equilibrium between antioxidants and oxidants in favour of oxidants (Sies, 1991). Oxidative stress might occur when the antioxidant defence system is overwhelmed by an increased oxidant burden or a reduced antioxidant supply.

More recently, the *reactive nitrogen species* (RNS) have been defined as a subgroup of oxidants deriving from nitric oxide ($NO^{\bullet}$) and the term *nitrosative stress* is used (for review, see Valko et al., 2007). The differentiation between ROS and RNS goes beyond the scope of the present review and for our purposes the term "oxidants" will include both ROS and RNS.

Generation and physiological roles of oxidants

Organisms are constantly exposed to exogenous and endogenous oxidants. Exposure to *exogenous oxidants* is of particular importance for the airways and the lungs, which are exposed to inhaled oxidants such as ozone, ultra-fine particles and endotoxins. Although the antioxidant defence system of the respiratory tract is particularly well developed (Mudway and Kelly, 2000), pulmonary oxidative stress appears as an important player in the pathogenesis of respiratory diseases (Rahman, 2005). In smokers, oxidants delivered by cigarette smoke contribute to oxidative stress, which is increased in patients suffering from chronic obstructive pulmonary disease (COPD) (Bowler et al., 2004). In equine respiratory research, ozone exposure has been shown to induce pulmonary oxidative stress (Deaton et al., 2005).

The *endogenous oxidants* can be divided into three groups depending on their origin. The major source of endogenous oxidants is the electron transfer chain of the mitochondria, where 1–3% of oxygen reduced into water may form superoxide ($O_2^{\bullet-}$) (Nivière and Fontecave, 1995; Kowaltowski and Vercesi, 1999). This pathway of oxidant formation is particularly important during exercise where oxygen consumption might increase up to 24 times in man and up to 40 times in horses (Art and Lekeux, 1993). Secondary contributors to endogenous oxidant generation are enzymes, such as xanthine oxidase, membrane oxidases, nitric oxide synthases, which physiologically produce oxidants (Nivière and Fontecave, 1995).

Although dependent on enzymatic activity, superoxide anion ($O_2^{\bullet-}$) generation by NADPH-oxidase during the "respiratory burst" of inflammatory cells can be considered a third important source of oxidants whose relative importance increases during inflammation-associated processes (Moslen, 1994). Cellular production and consequent liberation of oxidants by neutrophils and macrophages belong to the main stem of the non-specific immune response against invading micro-organisms. Oxidants play an important role by inactivating and destructing micro-organisms through peroxidation and destabilisation of their lipid membranes, oxidation and inactivation of their proteins acting as receptors or enzymes and oxidation of their nuclear material (Kowaltowski and Vercesi, 1999; Kobayashi et al., 2001).

Oxidant generation can be further enhanced in the presence of pro-oxidant elements, which transform oxidants into more reactive forms. For example, iron which favours transformation of hydrogen peroxide (H_2O_2) into hydroxyl radical ($HO^{\bullet}$), or myeloperoxidase (MPO), which transforms hydrogen peroxide into hypochlorous acid (HClO) (Roberfroid and Calderon, 1993a).

Informed opinion about the role of oxidants has changed over time. Initially, oxidants were considered to be potentially harmful by-products of cell metabolism, and as part of the immune system. However, during the last 20 years, they have become recognised as important messengers in numerous intra-cellular pathways. The so-called redox-signalling is implicated in the activation of transcription factors in mitotic and apoptotic processes, clearly indicating that oxidants are indispensable signalling molecules (Winrow et al., 1993; Valko et al., 2007). The expression of inflammatory genes in particular is dependent on oxidation-reduction reactions, which confers to oxidants the role of pro-inflammatory stimuli (Hensley et al., 2000).

Target molecules of oxidants and consequences of their oxidation

If exogenous or endogenous oxidant exposure increases or is insufficiently counterbalanced by antioxidants, oxidative damage occurs in form of oxidised DNA, proteins, lipids or carbohydrates. Virtually all cellular components may undergo oxidation in presence of high concentrations of oxidants, but the intra-cellular origin and the reactivity of the oxidant molecule, as well as the location and the biochemical properties of the target molecule play a determining role for the "pecking order" of oxidants (Buettner, 1993). This is the order according to which oxidant-antioxidant reactions occur.

Target molecules that have undergone oxidation can be detected and are used as oxidation markers. Their presence might disturb normal cell function, especially in the case of oxidative modifications to DNA, potentially leading to mutations, DNA strand breaks, etc. (Roberfroid and Calderon, 1993b). Oxidation of proteins can induce enzyme malfunction and cell membrane lipid peroxidation initiating chain reactions can compromise cell integrity (Lykkesfeldt and Svendsen, 2007). Such cellular perturbations appear to be of importance in inflammatory conditions,

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where increased oxidant production can further enhance the inflammatory process through a positive feedback mechanism, thereby inducing a vicious cycle (for review, see Valko et al., 2007). Consequently, molecules capable of counterbalancing this oxidant effect appear as potential tools to modulate such positive feedbacks.

The antioxidant defence system

Exposure to oxidants from a variety of sources has led organisms to develop a series of defence mechanisms, including preventative mechanisms, repair mechanisms, physical defences and antioxidant defences (Cheeseman and Slater, 1993). Exogenous reinforcement against the oxidative burden can be achieved by enhancing the antioxidant defence system, for example by increasing dietary antioxidant supply (Young and Woodside, 2001).

Prevention from oxidant generation

The prevention of oxidant generation occurs mainly within the mitochondrial respiratory chain where enzymatic complexes strongly limit electron leakage. Furthermore, proteins binding free iron and copper, such as transferrin, ferritin, ceruloplasmin or albumin, which favour oxidative processes, further decrease the cellular capacity of oxidant generation (Beckman and Ames, 1998).

Antioxidants

The major role of antioxidants is implied in the inactivation or transformation of oxidants, which can either be transformed by antioxidant enzymes into less reactive forms or which can react with antioxidant molecules that are chemically stable.

The most important antioxidant enzymes are superoxide dismutase (SOD), catalase (CAT) and glutathione-peroxidase (GPx) (Fridovich, 1995). The catalytic activity of these enzymes allows the transformation of superoxide anion into hydrogen peroxide (H_2O_2) and water, thereby inactivating important amounts of oxidants. Trace-elements, such as selenium (Se), zinc (Zn), copper (Cu) and manganese (Mn) play an important catalytic role for the enzymatic activity of GPx (Se) and SOD (Zn, Mn, Cu) (Maughan, 1999; Mates, 2000).

However, small antioxidant molecules are the most numerous endogenous antioxidants (for review, see Cheeseman and Slater, 1993). Except for lipoic acid, which acts as hydrophobic and hydrophilic antioxidant (Bast and Haenen, 2003), most endogenous antioxidants can be divided into two groups: (1) hydrophobic and (2) hydrophilic antioxidants. The former are mainly composed by α-tocopherol (vitamin E), β-carotene (vitamin A), flavonoids, ubiquinol (Coenzyme Q10), bilirubin and melatonin, and are essentially protecting lipids from chain reactions of peroxidation. On the other hand, the hydrophilic antioxidants, such as glutathione (existing under its reduced [GSH] and oxidised form [GSSG]), uric acid, ascorbic acid (vitamin C), thiols, proteoglycans and hyaluronic acid, protect against lipid peroxidation as well as against protein, carbohydrate and nuclear material oxidation. Fig. 1 displays the major oxidant pathways as well the most important antioxidants and their synergistic effects.

Repair mechanisms

If oxidation of cellular components has not been prevented by antioxidants, several mechanisms, inducing lysis or repair of oxidised proteins, lipids or nuclear material can be activated, thereby limiting functional repercussions of oxidative damage (Winrow et al., 1993; Beckman and Ames, 1998).

Diagnosis of oxidative stress: the assessment of the oxidant/antioxidant equilibrium

The generation of oxidants might vary as a function of their cellular origin, whereas the need for antioxidant

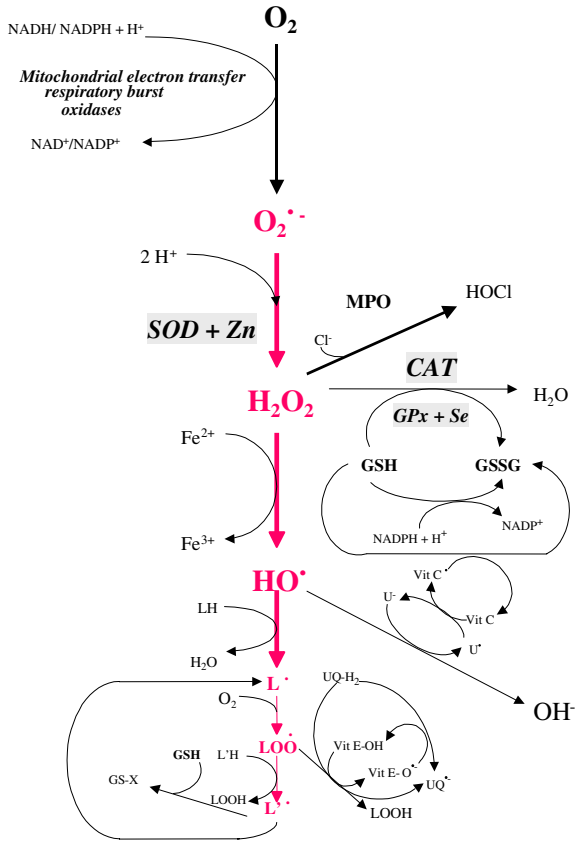

Fig. 1. Principal oxidative pathways occurring in living organisms and the main antioxidant systems transforming or inactivating free radicals or reactive oxidant species. O_2, oxygen; $O_2^{\cdot}$, superoxide anion; H_2O_2, hydrogen peroxide; HO•, hydroxyl radical; LH, fatty acid; L•, fatty acid radical; LOO•, lipid peroxide; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; MPO, myeloperoxidase; GSH, reduced glutathione; GSSG, oxidised glutathione; GS-X, glutathione linked to an oxidant; NADPH, nicotinamide adenine phosphate; U, uric acid; Vit C, vitamin C; Vit E, vitamin E; UQ, ubiquinone.

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defences depends on oxidant production and the efficiency of preventative mechanisms. This variability regarding the oxidant origin and antioxidant system being used has two consequences. Firstly, and as outlined in the review by Lykkesfeldt and Svendsen (2007), oxidative damage increases progressively when preventative, antioxidant and repair counteractions decrease or are overwhelmed. Consequently, the precise determination of an oxidative stress status, for example when irreversible modifications of cellular components due to oxidation are leading to cell dysfunction, remains challenging. Indeed, the measurement of oxidants, oxidant markers or antioxidants does not necessarily allow us to define oxidative stress. The characterisation of the ''oxidant/antioxidant equilibrium'' might therefore be a more appropriate term because it is likely that in many conditions where oxidants and antioxidants are determined, oxidative stress may not occur or only to a limited extent.

The second consequence concerns the choice of the oxidants, oxidant markers or antioxidants being measured as this is of great importance in gauging the meaning of the oxidant/antioxidant equilibrium. Although there are many factors limiting the number of oxidant markers and antioxidants that can be investigated, such as reduced sample volumes and cost limitations, care should be taken to perform a global approach to the oxidant/antioxidant equilibrium taking into account target molecules of different classes (proteins, lipids, DNA) and different antioxidants (enzymes, lipophilic and hydrophilic antioxidants).

The general approach of the oxidant/antioxidant equilibrium is based on the assessment of free radicals, antioxidants, pro-oxidant elements and oxidant markers. Free radical detection allows the identification and localisation of oxidants, but necessitates very complex equipment that is rarely available. Measurement of oxidative markers, used as ''fingerprints'' of oxidant action, is mostly practised, as well as determination pro-oxidant elements and antioxidants (for reviews, see de Zwart et al., 1999; Tsimikas, 2006).

The biological matrix or the organ selected for the establishment of an oxidant/antioxidant profile might impose some limits with regard to sample availability and will also influence the markers and antioxidants being investigated. Table 1. summarises the most important sampling materials used in horses and indicates limiting factors as well as the most frequently used markers.

Another crucial point for successful oxidant/antioxidant equilibrium assessment is the rapid and appropriate processing and storage of collected samples. As oxidative processes continue and even increase by exposure to ambient oxygen, ambient temperature and UV light, samples must be cooled immediately after collection and should be protected from light. Sample processing needs to be performed rapidly (<2 h after collection) and should sometimes include the addition of stabilising agents and/or centrifugation prior storing. The storage conditions required might vary depending on the marker selected: at 4 °C, at −20 °C, at −80 °C or even in liquid nitrogen. Some markers degrade rapidly irrespective of storage conditions and need to be analysed within several hours of collection. A careful planning of oxidant/antioxidant equilibrium assessment in research or clinical conditions is therefore important.

The oxidant/antioxidant equilibrium in horses

During the last decade more than 100 studies describing the oxidant/antioxidant equilibrium in the equine species have been published. While a large part of this work is fundamentally descriptive, other studies have compared pathological with physiological conditions. However, only a few studies have tested whether antioxidant supplements, mostly administrated orally, improved the oxidant/antioxidant status and affected clinical parameters.

We will now consider the most frequently investigated pathological conditions where the oxidant/antioxidant equilibrium has been addressed, and summarise current knowledge on the oxidant/antioxidant equilibrium in equine reproduction and equine sports medicine. The most important antioxidant supplementation trials that have been undertaken in sport horses will also been reviewed.

The oxidant/antioxidant equilibrium in pathological processes

Lower airway diseases

Horses suffering from *recurrent airway obstruction* (RAO) or heaves are subject to pulmonary and systemic oxidative stress, which has been demonstrated by increased levels of GSH, GSSG, 8-isoprostane, MPO or decreased levels of ascorbic acid in pulmonary epithelial lining fluid (PELF) and increased levels of GSH and GSSG in erythrocytes (Art et al., 1999; Kirschvink et al., 2002a; Deaton et al., 2004a; Art et al., 2006b). Hydrogen peroxide (H_2O_2) in exhaled breath condensate has also been assessed and has been correlated with ascorbic acid concentration in PELF (Deaton et al., 2004b).

The pulmonary oxidative markers and antioxidants are correlated with the intensity of airway inflammation, essentially with the neutrophil percentage of bronchoalveolar lavage fluid (BALF), which suggested that the inflammatory burst is the main contributor to increased ROS production (Kirschvink et al., 2002a; Deaton et al., 2004a,b; Art et al., 2006b). Significant correlations between lung function variables (ventilatory mechanics and arterial blood gas) and oxidant markers have been detected in a study comparing healthy horses with RAO-affected horses in clinical remission and during exacerbation (Kirschvink et al., 2002a), which indicated that pulmonary oxidative stress could have an impact on lung function. Most of these studies investigated the pulmonary oxidant/antioxidant status of the horses under resting conditions as well as after a standardised exercise test (Art et al., 1999; Kirschvink et al., 1999, 2002a).

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Table 1
Overview of biological matrixes and conditions that are of interest for oxidant/antioxidant equilibrium assessment in horses

Biological matrix	Pathological condition	Limiting factor	Frequently assessed markers	Studies determining at least one marker
Red blood cells or whole blood	Exercise-induced oxidative stress, grass sickness	–	CAT, GPx, SOD GSH, GSSG	(McMeniman and Hintz, 1992) (Chiaradia et al.,1998) (Frankiewicz-Jozko and Szarska, 2000) (McGorum et al., 2003) (de Moffarts et al., 2004, 2005a,b) (Williams and Carlucci, 2006)
White blood cells	Exercise-induced oxidative stress	–	GPx, GSH apoptosis	(Avellini et al., 1999) (Williams et al., 2004a,b)
Plasma/serum	Exercise-induced oxidative stress, grass sickness, equine motor neuron disease	–	Vitamin A, E, C Antioxidant capacity of plasma 4-hydroxynonenal (4-HNE) LPO MDA MPO Protein carbonyls TBARS Trace-elements (Se, Cu, Mn)	(McMeniman and Hintz, 1992) (Siciliano et al., 1997) (Chiaradia et al., 1998) (Frankiewicz-Jozko and Szarska, 2000) (White et al., 2001) McGorum et al., 2003) (de Moffarts et al., 2004) (Kinnunen et al., 2005a,b) Williams et al., 2004a,b; Williams et al., 2005a,b (McGorum et al., 2006) (Art et al., 2006a) (Williams and Carlucci, 2006)
Muscle biopsy	Exercise-induced oxidative stress	Invasiveness, sample size	GPx GST Protein carbonyls TBARS	(McMeniman and Hintz, 1992) (Kinnunen et al., 2005a,b)
Exhaled breath	Airway disease, Exercise-induced oxidative stress	Complexity of collection device	Volatile substances (pentane, ethane etc.)	(McMeniman and Hintz, 1992) (Wyse et al., 2004)
Exhaled breath condensate	Airway disease	Only small molecular weight markers due to collection device	H_2O_2	(Deaton et al., 2004b)
Tracheal and bronchoalveolar lavage fluid	Airway disease	Variable dilutions due to sampling technique	GSH-GSSG, UA, 8-isoprostane Vit C, Vit E MPO	(Kirschvink et al., 2002a) (Deaton et al., 2004) (Art et al., 2006a)
Synovial fluid	Joint disease	Volume	Protein carbonyl Nitrotyrosine 8-Isoprostane	(Dimock et al., 2000) (Van der Harst et al., 2006) (Daix et al., 2007)

CAT: catalase; GPx: glutathione peroxidase; GSH: glutathione (reduced form); GSSG: glutathione (oxidised form); GST: glutathione-s-transferase; LPO: lipid peroxides; MDA: malondialdehyde; MPO: myeloperoxidase; SOD: superoxide dismutase; TBARS: thiobarbituric acid reactive substances; UA: uric acid.

Exercise itself has been shown to induce pulmonary oxidative stress in horses and human athletes and can therefore further increase the pulmonary oxidant/antioxidant disequilibrium in the equine athlete. In the particular case of summer-pasture associated obstructive pulmonary disease, increased inducible nitric oxide synthase (iNOS) has been seen in bronchial epithelial cells of affected horses, indicating that nitric oxide (NO) might play a pro-inflammatory role in this pathological condition (Costa et al., 2001).

Based on these findings and on similar observations made in human patients suffering from asthma or chronic obstructive pulmonary disease (COPD) (Rahman et al., 2000; Rahman, 2005), oral antioxidant supplementation trials providing vitamin E, vitamin C (ascorbic acid), selenium and natural flavonoids have been performed in RAO-affected horses (Deaton et al., 2002; Kirschvink et al., 2002b). The results showed that vitamin and trace-element supplementation significantly increased the blood concentrations of these substances and that PELF ascorbic acid concentrations can be increased (Deaton et al., 2002). However, no beneficial effects on other antioxidants, such as GSH, or on oxidative markers, such as 8-isoprostane, could be evidenced (Kirschvink et al., 2002b). From a functional point of view, an improved bronchoscopy score was detected in supplemented horses, but no effect on the functional variables assessed in this trial were detected (Kirschvink et al., 2002b).

As outlined by Deaton (2006), further studies, including a large panel of oxidant and antioxidant markers and

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functional tests evaluating the degree of airway obstruction, airway hyperresponsiveness and mucus hypersecretion, are necessary in order to establish a better relationship between oxidant/antioxidant disequilibrium and the clinical manifestations of RAO. In human respiratory research, clinical trials with antioxidants showed controversial results with regards to the oxidant/antioxidant status, whereas epidemiological studies suggest that increased vitamin intake has beneficial effects on lung function or diminishes exacerbations of COPD (Rahman et al., 2000). Consequently, it can be concluded that good quality antioxidant supplementation might be helpful for improving the pulmonary oxidant/antioxidant equilibrium RAO-horses, possibly by decreasing the ROS-dependent pro-inflammatory stimulus.

Exercise-induced pulmonary haemorrhage (EIPH) has also been related to an oxidant/antioxidant disequilibrium. As oxidants might decrease $NO^{\cdot}$ levels and increase the synthesis of vasoconstrictor agents, pulmonary vascular pressure (considered as the primary cause of EIPH) may be further increased and result in the rupture of pulmonary capillaries (Mills and Higgins, 1997). This hypothesis has however been challenged and remains to be proved (Kindig et al., 2001).

Another factor that could have an impact on EIPH is exercise-induced erythrocyte stiffness. It has been shown that erythrocyte membrane fluidity significantly decreases in response to strenuous exercise and that correlations with oxidative markers exist (Baskurt and Meiselman, 1999; de Moffarts et al., 2006; Portier et al., 2007). Erythrocyte stiffness could contribute to increased pulmonary pressures by increasing blood viscosity. Further studies aimed at reducing exercise-induced erythrocyte stiffness would be interesting, not only in the field of EIPH, but also in other conditions where perfusion disorders are associated with an oxidant/antioxidant disequilibrium.

Neurological disorders

An oxidant/antioxidant disequilibrium is believed to play a role in the pathophysiology of equine grass sickness (dysautonomia) (McGorum et al., 2000, 2003), equine motor neuron disease (EMND) (Hahn and Mayhew, 1997; de la Rua-Domenech et al., 1997; McGorum et al., 2006; Divers et al., 2006) and equine degenerative myeloencephalopathy (Blythe et al., 1991). Although the aetiopathogenesis of these disorders is still poorly understood, oxidant-related disorders of the neural function might be implicated. It has been hypothesised that an increase in glucose metabolism could be the potential cause of the decreased vitamin E plasma concentration detected in horses with EMND (van der Kolk et al., 2005).

In human medicine, oxidative stress is actually associated with many neurological disorders, such as Alzheimer's disease, Parkinson's disease (Montine et al., 2001) or amyotrophic lateral sclerosis (Mitchell and Borasio, 2007). Indeed, amyotrophic lateral sclerosis or "motor neuron disease" shares some features with EMND and a mutation of the gene coding for SOD-1 appears to be implied in its aetiopathogenesis (Mitchell and Borasio, 2007). Nervous tissue contains important amounts of lipids and is highly sensitive to lipid peroxidation. Equine Cushing's syndrome, which is due to dysfunction of the pars intermedia of the pituitary gland, could also be dependent on a primary neurological oxidant disorder (Keen et al., 2004).

Muscle disorders

White muscle disease in foals is primarily due to selenium-vitamin E deficiency and is often associated with inappropriate physical exercise or increased dietary supply of unsaturated lipids (Lofstedt, 1997). Steatitis, also named yellow fat disease, polymyositis or muscular dystrophy, could also be related to nutritional selenium and/or vitamin E deficiencies (Foreman et al., 1986). Seasonal pasture myopathy reported in USA (Finno et al., 2006) and atypical myopathy reported in Europe (Votion et al., 2006) also seem to be associated with an oxidant/antioxidant disequilibrium, although it is unlikely that it is the primary cause.

A clear relationship between exertional rhabdomyolysis (tying-up syndrome) and vitamin E or selenium deficiency has not yet been described in horses (Valentine et al., 1998) and could not be demonstrated in sled dogs (Piercy et al., 2001). Nevertheless, these conditions are treated by aggressive antioxidant therapy (vitamin E, selenium, vitamin C).

Perfusion-related disorders

Equine post-anaesthetic myositis or myopathy usually occurs in large horses that have undergone prolonged anaesthesia. Compression of the muscle masses occur during recumbency and post-ischaemic hyperaemia, leading to increased ROS release, and the condition can be observed up to a week after muscle reperfusion (Serteyn et al., 1988, 1990). It is likely that massive ROS release induces local membrane lipid peroxidation, leading to lesions of the muscle cell membrane and myopathy.

Ischemia and reperfusion processes also occur during colic in horses and an increased release or ROS after resolution of an intestinal torsion for example could be expected. In experimentally induced ischemia-reperfusion in the jejunum and large colon of horses, significant oxidant/antioxidant disorders were detected in the jejunum, but not in the colon (Kooreman et al., 1998). However, plasma MPO levels measured in horses suffering from large intestinal obstruction were increased as a function of strangulation severity, indicating that leukocyte activation and ROS release occurred during colonic ischemia (Grulke et al., 1999).

Laminitis is also cited among perfusion-related disorders in horses. If it is clearly established that an important inflammatory reaction develops during the prodromal stage of laminitis, it is less clear whether ROS play a primary role in the pathophysiology of this disease. Loftus et al. (2007) showed that the equine digital laminae have

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a relatively limited SOD activity, which might render this tissue highly susceptible to oxidant-mediated injury.

Perfusion-related disorders with or without underlying inflammatory reaction can disturb the oxidant/antioxidant equilibrium. Therefore, antioxidants and free radical scavengers such as dimethylsulfoxide (DMSO), Carolina rinse and allopurinol are frequently recommended as preventive and/or therapeutic agents (Sykes and Furr, 2005; Soffler, 2007).

Joint diseases

In human medicine, the oxidant/antioxidant disequilibrium is believed to play an important role in the development of degenerative joint disease (Aigner et al., 2006). In horses, only a few studies investigating the oxidant markers in synovial fluid or synoviocytes are available. Elevated concentrations of protein carbonyl have been found in synovial fluid of horses with joint disease (Dimock et al., 2000), as well as increased concentrations of the lipid peroxidation marker 8-isoprostane (Daix et al., 2007). Nitrotyrosine and nitrite in cartilage and subchondral bone are increased in horses suffering from osteoarthritis (van der Harst et al., 2006). It appears however that age-related metabolic bone or cartilage activity can influence these markers, which necessitates careful comparisons between young, healthy and diseased horses.

Spin trapping techniques have shown that cultured synoviocytes, but not chondrocytes, increase ROS formation after repeated anoxia/re-oxygenation cycles, which could possibly favour the onset of osteoarthritis (Schneider et al., 2005).

The oxidant/antioxidant equilibrium in physiological processes

Equine reproduction

In human reproductive biology and endocrinology, ROS are recognised as key signal molecules in physiological processes, such as oocyte maturation and fertilisation, pregnancy and parturition. An oxidant/antioxidant imbalance seems to be involved in the onset of infertility and in numerous pathological conditions, such as abortion, eclampsia and endometrioses (for review, see Agarwal et al., 2005). In equine medicine, oxidant/antioxidant research has focused primarily on male reproduction. Indeed, among the factors affecting semen viability, motility and semen plasma membrane function involve oxidants and antioxidants (Baumber et al., 2000, 2002). ROS and cryopreservation promote DNA fragmentation in equine spermatozoa (Baumber et al., 2003). Particularly in the case of cooled-stored stallion semen, antioxidants play an important role in the composition of semen extenders (Aurich, 2005; Kankofer et al., 2005; Pagl et al., 2006).

Exercise and training

Since Davies and co-workers demonstrated in 1982 that physical exercise generates free radicals, the impact of exercise and training on the oxidant/antioxidant equilibrium has been widely investigated in laboratory rodents (for review, see Leeuwenburgh and Heinecke, 2001), in humans (for review, see Sen and Packer, 2000) and in horses. Exercise-induced changes of the oxidant/antioxidant equilibrium are essentially due to increased mitochondrial electron transport within muscle cells (DiMeo and Venditti, 2001). An increased ROS generation could favour membrane peroxidation of muscle cells and thereby decrease their membrane integrity. Positive correlations that have been detected between muscle enzyme leakage and plasma lipid peroxides support this hypothesis (Van Erck, personal communication; Williams et al., 2004a).

Numerous studies have shown that exercise-induced oxidant/antioxidant changes in exercising horses vary with regard to exercise type (race, standardised treadmill exercise, standardised race track exercise, endurance) and the markers assessed in blood, although it is generally agreed that exercise does induce significant alterations of the circulatory oxidant-antioxidant balance. However, some controversy exists in terms of poorly reproducible and even contradictory results that suggest that experimental design, the horses' fitness, the analytical approach and environmental factors strongly influence the study results. An overview of the most representative studies performed in this field is given in Table 2.

It appears that exercise intensity (monitored by oxygen consumption or heart rate), exercise duration and atmospheric conditions (temperature, relative humidity) are determining factors for this exercise-induced pro-oxidant burden (Mills et al., 1996; Williams et al., 2005a).

Another interesting finding is the fact that exercise-induced changes of antioxidants do not necessarily appear during or immediately after exercise, but that they can be detectable 16–24 h later (Balogh et al., 2001; de Moffarts et al., 2004; Marlin et al., 2002). It has not yet been addressed whether such prolonged perturbations of the oxidant/antioxidant equilibrium influence consecutive bouts of exercise, such as training. However, training might positively influence the antioxidant capacity of the organism. Indeed, an increase in different antioxidants, such as vitamin C, uric acid, GPx and SOD, has been observed in Standardbreds after a 12-week period of aerobic and anaerobic training, while the increase in oxygen consumption (measured as change of VO_2 max) was positively correlated with the increase of erythrocyte SOD activity (de Moffarts et al., 2004).

Although training increases the antioxidant defence system of the organism, prolonged periods of training and racing competitions may induce disturbances of the oxidant/antioxidant equilibrium (Avellini et al., 1995; de Moffarts et al., 2005a), which could be related to inappropriate nutritional antioxidant supply. In human sports medicine, it has been established that the need for trace-elements and vitamins is increased in athletes (Clarkson and Thompson, 2000; Jenkins, 2000). Rats that were depleted of GSH have been shown to be less exercise tolerant than

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Table 2
Overview of studies investigating the effect of exercise on the oxidant/antioxidant equilibrium

Horses (*n*) and exercise test	Evolution of blood markers (pre- versus post-exercise)	References
Untrained Thoroughbreds ($n = 4$)/strenuous treadmill exercise	Increase of *urinary* MDA and hydroxyproline 24 h post-exercise	(Mills et al., 1994)
Thoroughbreds ($n = 11$)/race track test	Increase of plasma TBARS, antioxidant capacity, total antioxidant reactivity 5 min after end of race	(Avellini et al., 1999)
Thoroughbreds ($n = 6$)/treadmill exercise with various ambient T° and relative humidity	Increased plasma lipid peroxides, UA, and RBC GSSG, decreased iron-binding antioxidant activity of plasma immediately after exercise	(Mills et al., 1996)
Thoroughbreds ($n = 30$)/race track test	Increased plasma TBARS, PAOC, TAR 5 min post-exercise	(White et al., 2001)
Eventing horses ($n = 14$)/simulated jumping competition	Increased plasma UA 5 min post-exercise; decreased TBARS and increased RBC GSH at 24 h post-exercise	(Balogh et al., 2001)
Endurance horses ($n = 5$)/160 km race	Decreased plasma total antioxidant status and increased whole blood GPx after 60 km, increased serum TBARS and whole blood SOD after exercise	(Frankiewicz-Jozko and Szarska, 2000)
Endurance horses ($n = 35$)/80 or 160 km race	Decrease of plasma Vitamin C, RBC GSH; increase of RBC GPx during and after exercise	(Hargreaves et al., 2002a)
Endurance horses ($n = 30$)/80 km race	Decreased RBC GSH and plasma Vitamin C, unchanged plasma Vitamin E and RBC GPx	(Hargreaves et al., 2002b)
Endurance horses ($n = 40$)/140 km race	Decreased RBC GSH and GSSG at end of exercise; increased TBARS at end of exercise; decreased RBC GSH, GSSG, GRR and plasma AA 16 h post-exercise	(Marlin et al., 2002)
Endurance horses ($n = 34$, 11, 24 respectively)/three 80 km races	Increase of plasma lipid peroxides, decline of RBC GPx and plasma tocopherol immediately after end of exercise; reduced between-race repeatability	(Williams et al., 2005a)
Standardbred ($n = 6$)/treadmill exercise	Increase of plasma Vitamin C and UA, increase of RBC GRR%; decrease of Vitamin A and GSH during and/or immediately after end of intense exercise	(de Moffarts et al., 2004)
Standardbred ($n = 12$) versus Eventing horses ($n = 12$)/field exercise tests	Increase of plasma Vitamin C, UA, ACW; decrease of RBC GSH and plasma lipid peroxides 15 min post-exercise	(de Moffarts et al., 2005b)
Standardbred ($n = 8$)/treadmill exercise	Increased plasma and *muscle* carbonyl proteins, unchanged 4-hydroxynonenal protein adducts and unchanged HSP expression 4 h after a moderate exercise	(Kinnunen et al., 2005a)
Standardbred ($n = 8$)/treadmill exercise	Increased plasma lipid peroxides 4 h after moderate intensity exercise	(Kinnunen et al., 2005b)
Standardbred ($n = 12$)/treadmill exercise	Increased plasma retinol and beta-carotene, increased RBC GSH and GPx immediately after intense exercise	(Williams and Carlucci, 2006)
Eventing horses ($n = 12$)/race track exercise	Increase of plasma ACW, UA, lipid peroxides 15 min post-exercise; increase in RBC GSH and plasma oxidised proteins 24 h post-exercise	(de Moffarts et al., 2006)
Eventing ponies ($n = 9$)/3-day event competition	Increase of plasma MPO 30 min after cross country competition	(Art et al., 2006)

ACW: antioxidant capacity of water soluble components; GSH: reduced glutathione; GSSG: oxidised glutathione; GRR: glutathione redox ratio (GRR = GSSG/(GSH + GSSG); MDA: malondialdehyde; MPO: myeloperoxidase; RBC: red blood cell; PAOC: plasma antioxidant capacity; TBARS: plasma thiobarbiturate reactive substances; TAR: total antioxidant reactivity; UA: uric acid.

non-depleted or supplemented rats, suggesting that an antioxidant deficiency decreases performance, whereas antioxidant supplementation in non-deficient individuals does not improve performance (Sen et al., 1994). Accordingly, antioxidant supplements appear to be of interest in preventing an exercise-induced (or disease-induced) deficiency, but supplementation does not seem to improve performance.

Antioxidant supplementation trials in sport horses

Antioxidant trials performed in sport horses provide evidence that exercise-induced disturbances or deficiencies can be at least partially prevented and there are a number of significant antioxidant trials that will now be considered.

Vitamin E supplementation: The first report on the effect of vitamin E status on lipid peroxidation in exercised horses was published in 1992 (McMeniman and Hintz, 1992). Oral vitamin E supplementation of polo ponies undergoing treadmill exercise tests was performed and the relationship between blood vitamin E concentration and lipid peroxidation (assessed by thiobarbituric acid reactive substances; TBARS) was assessed. Supplementation in the form of corn oil increased plasma vitamin E concentration and a negative correlation between plasma vitamin E and TBARS levels was found for pre- and post-exercise. Interestingly, increased activity of whole blood GPx and SOD and an increase in plasma vitamin C were recorded after vitamin E supplementation, which suggested to the authors that the polyunsaturated fatty acids provided by corn oil increased the pro-oxidative burden irrespective of vitamin E supplementation. Alternatively, it could be hypothesised that vitamin E supplementation increased antioxidant recycling and improved synergistic antioxidant effects.

Another vitamin E supplementation study investigated the effect of three diets that provided different levels (deficient – low – high) of vitamin E over 90 days in 19 regularly exercised horses (Siciliano et al., 1997). Although serum and muscle vitamin E concentrations increased in horses receiving the low or the high vitamin E regimen, muscle TBARS concentrations remained unaffected, as well as

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serum creatine kinase (CK) and aspartate aminotransferase (AST) activities.

The study of Avellini et al. (1999) investigated the effect of vitamin E in combination with selenium in 11 racehorses that underwent a 70-day training and supplementation period. Here, training combined with supplementation induced a significantly lower exercise-induced serum malondialdehyde (MDA) generation and the total peroxyl-radical trapping (TRAP) capacity of plasma was significantly increased. Vitamin E and MDA levels were, as with vitamin E and TBARS in the study of McMeniman and Hintz (1992), significantly and negatively correlated. Although this study did not allow dissociation between training and supplementation effects, it demonstrated a clear modulation of the oxidant/antioxidant equilibrium, which is in agreement with the study of de Moffarts et al. (2004), who investigated the effect of exercise intensity and training in Standardbred horses.

Very intense vitamin E supplementation of horses has failed to improve modulation of exercise-induced oxidative changes in comparison with basal supplementation (Williams and Carlucci, 2006). Moreover, plasma vitamin A (β-carotene) concentration decreased, possibly due to an inhibitory effect of vitamin E on vitamin A metabolism.

Vitamin C supplementation: The effect of vitamin C supplementation has been investigated in racehorses (White et al., 2001) and endurance horses (Williams et al., 2004a). In the first study, 5 g of vitamin C were intravenously injected to 14 Thoroughbred horses prior to a race, and 30 horses ran the race without any treatment. Vitamin C increased plasma vitamin C levels, prevented exercise-induced increase in plasma TBARS and maintained the plasma antioxidant capacity and total antioxidant reactivity; serum CK activity increased in both treated and untreated horses. In the second study, 46 endurance horses were supplemented for 3 weeks prior to an 80-km endurance race with either vitamin E ($n = 23$) or a combination of vitamin E and vitamin C ($n = 23$) (Williams et al., 2004a). Whereas vitamin C levels remained higher in horses receiving both antioxidants, no treatment-related differences regarding lipid peroxides, red blood cell GSH, red und white blood cell GPx activity, serum CK and AST activity were reported. Positive correlations between plasma lipid peroxides, CK and AST activities were however detected, suggesting an association between muscle leakage and exercise-induced lipid peroxidation.

Lipoic acid versus vitamin E supplementation: In a study comparing non-supplemented control horses ($n = 4$) with vitamin E-supplemented ($n = 4$) or lipoic acid-supplemented endurance horses ($n = 4$), red and white blood cell GPx activity, white cell apoptosis, whole blood GSH, plasma CK, AST, lactic acid, vitamin C and E were assessed after 2 weeks of oral supplementation immediately before, during and after a 55-km endurance race (Williams et al., 2004b). Lipoic acid- and vitamin E-supplemented horses had both higher plasma vitamin E and vitamin C levels than control horses, as well as higher whole blood GSH concentrations and white blood cell GPx activity, whereas whole blood GPx activity was lower. These results illustrated the recycling and scavenging effect of lipoic acid (Bast and Haenen, 2003) and provided evidence that the modulation of the glutathione system, which is of primary importance of the antioxidant system of erythrocytes and muscle cells (Ji et al., 1998), can be achieved in horses. Moreover, this report showed that the exercise-induced increase of lactic acid and CK was lower in supplemented horses, whereas AST activity was only lower in lipoic acid-supplemented animals. Exercise-induced white blood cell apoptosis was, however, decreased in both supplemented groups.

Vitamin and trace-element combination: In a field study investigating 40 trained Thoroughbreds over a racing period of 3 months, 30 horses received a daily supplement containing vitamin E, C, A, copper, zinc and selenium (de

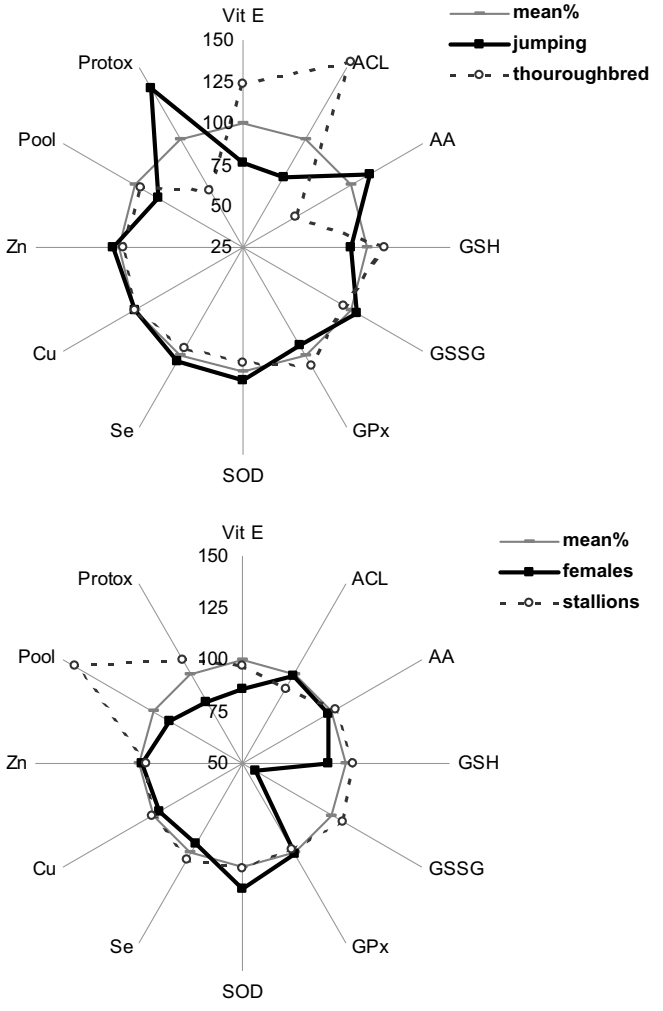

Fig. 2. Illustration of breed- and gender-related differences of the oxidant/antioxidant equilibrium in horses. Data are shown as percentages in comparison to mean values. ACL, antioxidant capacity of lipid soluble substances in plasma; ACW, antioxidant capacity of water soluble substances in water; GSH, reduced glutathione; GSSG, oxidised glutathione; GPx, glutathione peroxidase; SOD, superoxide dismutase; Se, selenium; Cu, copper; Zn, zinc; Pool, lipid peroxides; Protox, oxidised proteins.

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Moffarts et al., 2005a). The oxidant/antioxidant equilibrium was assessed at rest before, and 6 and 12 weeks after the supplementation period. In comparison to the placebo, the supplement significantly increased plasma vitamin E, vitamin A and selenium concentrations, as well as whole blood GPx activity and plasma lipophilic antioxidant capacity. However, no effect on whole blood GSH, which decreased, and on GSSG, which increased, was noted in comparison to the placebo group.

On the basis of the literature dealing with exercise-induced oxidative changes and the variable success of antioxidant supplementation, it is currently not yet possible to recommend an antioxidant supplementation programme that would be appropriate for every horse. Indeed, gender-, age- and breed-related differences of several oxidant/antioxidant markers and biochemical markers have been demonstrated in a study including 493 clinically healthy sport horses (Kirschvink et al., 2006). Fig. 2 shows a comparison between relative mean values of markers that were assessed in jumping horses and Thoroughbreds as well as between males and females.

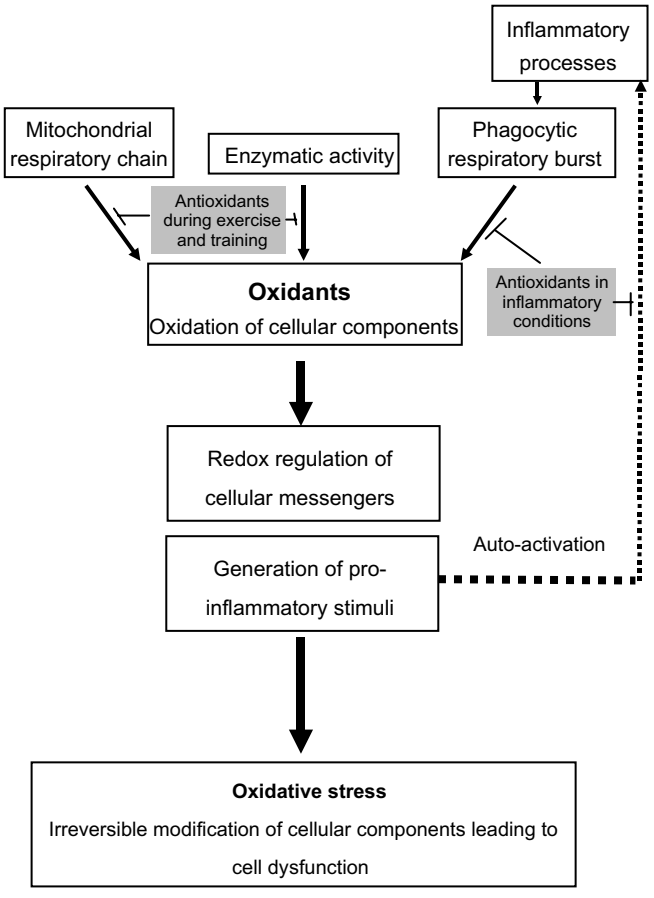

Fig. 3. Main sources of oxidants and consequences of oxidation processes leading to oxidative stress and level of action of antioxidant agents.

Conclusions

Should antioxidants be used and how should they be used?

Disturbances of the oxidant/antioxidant equilibrium are seen in many pathological and physiological conditions and appropriate antioxidant supplementation may have beneficial effects by protecting cell components against oxidation and/or by restoring the redox signalling that is implicated in inflammatory cascades (Fig. 3).

Antioxidant therapy can be considered helpful in the treatment of certain pathological conditions, particularly chronic inflammatory conditions, but should be combined with other therapeutic approaches. In the case of sport horses, individually adapted antioxidant supplementation may be useful in improving general health and in maintaining physical fitness. However, prior to antioxidant supplementation, the basal nutritional supply in terms of energy, carbohydrates, proteins, lipids and minerals of the animals should be verified and, if necessary, adapted to the animal's needs. Table 3 indicates some specific needs for vitamins and trace-elements for different horse groups.

All antioxidant vitamins are susceptible to oxidation and have a limited half-life. As a result they can undergo oxidation during production or prolonged storage. The bioavailability of trace-elements may vary in the presence

Table 3
Recommended daily provision in vitamins and trace-elements for horses

Vitamin	Unit	Training	Light work	Pony	Mare (pregnant)	Foal	Yearling
A	IU	60000	50000	18000	40000	12000	36000
D	IU	6000	5000	2500	4000	1000	2500
E	IU	2000	1200	600	1000	200	600
K	mg	10	6	3	4	2	4
C (ascorbic acid)	mg	1500			600	400	
B1	mg	80	50	25	32	12	25
B2	mg	30	15	9	12	6	9
B6	mg	30	18	9	12	6	9
B12	mg	1.2	0.8	0.1	0.12	0.08	0.08
Niacin	mg	100	60	30	40	36	36
Pantothenic acid	mg	45	27	12	16	10	15
Biotin	mg	1.5	1	0.45	0.8	0.5	0.6
Folic acid	mg	100	50	15	20	20	15
Choline	mg	800	600	250	400	250	300
Trace-elements							
Cobalt	mg	7.5					
Copper	mg	100					
Manganese	mg	600					
Iron	mg	600					
Iodine	mg	1.5					
Selenium	mg	2.5					
Zinc	mg	600					

IU: international units. Where no data are indicated, values given in the first column should be considered. These reference values have been compiled from the following references: Wolter (1985), Anonymous (1989), Hintz (1987), Kohnke et al. (1999), Pagan (2000).

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of other, possibly inappropriate, amounts of minerals or other trace-elements. Consequently, the quality and efficiency of commercialised supplements may vary considerably and a careful selection is recommended.

Although the concentrations of vitamins or trace elements that are supplemented can be rapidly detected in the blood, the beneficial effect on the oxidant/antioxidant equilibrium (and in some cases on clinical variables) should only be expected after a few weeks. Most antioxidant supplementation trials cover a period of 4–8 weeks before potential effects are evaluated. In the case of intensively exercising sport horses or horses suffering from a chronic inflammatory condition, prolonged or even permanent supplementation can sometimes be necessary because these animals have a persistent and increased need for antioxidants.

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