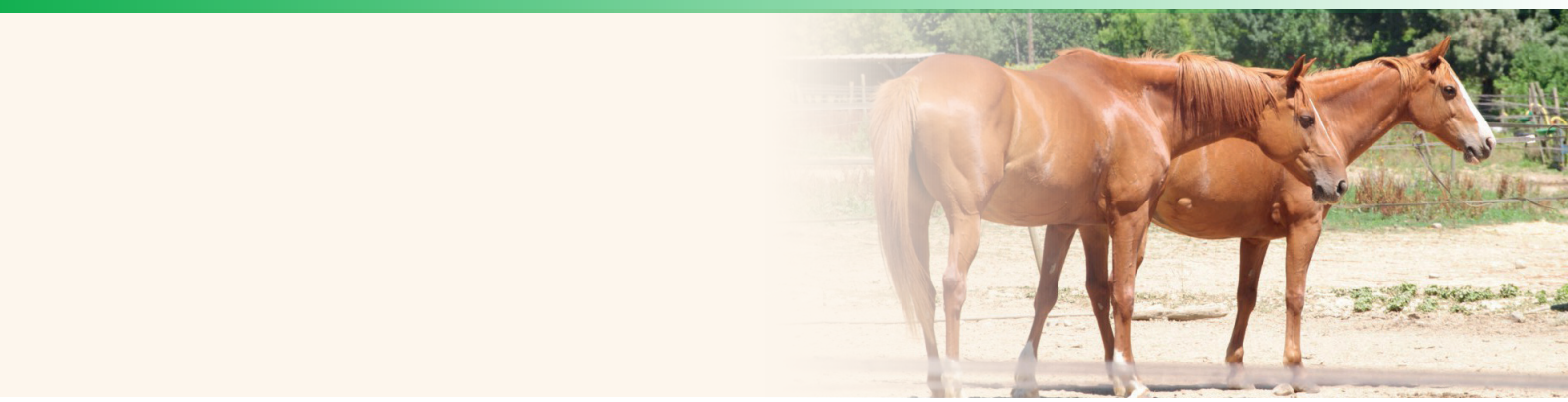
SCIENTIFIC REVIEW



Muscle and Joint Integrity: Current Situation, Prevention and Benefits of Primary Antioxidants

Benefits of MELOFEED & ALKOSEL Antioxidants

alkosel The Premium Source of Bioavailable Selenium

melofeed NATURAL SOURCE OF PRIMARY ANTIOXIDANTS

Table of Contents

Introduction

The qualities of "strength" and "speed" innate to horses as a species are often exploited to their maximum to optimise performance, meaning competition horses are regularly pushed to their physiological limits. Joints and muscles are key elements in the performance of competition horses. However, intense physical activity is often underestimated as a major source of oxidative stress, which is linked to excessive production of reactive oxygen species (ROS), and as a source of inflammation, which impairs the resistance of the horse's muscles and joints.

Indeed, oxidative stress leads to an exacerbated inflammatory response that can jeopardise the natural regeneration of damaged tissue. Although inflammation is part of the normal reaction to stress (during heavy exertion, sustained physical exercise or in a competition), this physiological phenomenon must be controlled so that slightly damaged tissues are able to regenerate properly. In equids, oxidative stress, which is very common in working horses, is recognised as often being associated with the weakening of the membranes of muscles and joints. Violent physical exercise leads to micro-trauma that will damage the ultrastructure of muscle fibres and encourage local infiltration by white blood cells (onset of the inflammatory response), rapidly leading to a state of oxidative stress. The problem is how to regulate and balance the intensity of the inflammatory response, which although potentially responsible for cellular damage is absolutely necessary for the healing process.

Various indicators are described in the literature for objectively measuring the limits of resistance and even detecting critical situations in which physiological thresholds are exceeded. These blood and synovial biomarkers are accurate indicators of muscle and joint integrity and are therefore useful predictors of a horse's sporting future.

1- Specificities and challenges of the equine muscle and joint integrity

1-1 Muscle integrity

ANATOMICAL REVIEW OF THE HORSE MUSCLE SYSTEM

Horses have 469 muscles responsible for bone movement. The muscle mass represents half of a horse's live weight. Muscles consist of a set of fibres that act by extension or contraction. They may be directly attached to bones or attached by means of tendons. Muscles play various roles in locomotion (extension, flexion, rotation, abduction and adduction) and are classified in 2 categories:

- Striated muscles or red muscles are responsible for locomotion and work by voluntary contraction
- Smooth muscles or white muscles are essential for the proper functioning of vital processes such as breathing, or the normal function of the digestive tract. These muscles work by involuntary contraction.

This paper is solely concerned with striated muscles.

MECHANISMS RESPONSIBLE FOR ALTERING MUSCLE FUNCTION FOLLOWING OXIDATIVE STRESS

Oxygen (O_2) is essential for the survival of all aerobic organisms. In living beings that require oxygen molecules in order to live or function, roughly 85-90% of the cellular oxygen is used by the mitochondria to produce energy in the form of a molecule called ATP (*adenosine triphosphate*) in the cellular respiration reaction. However, during this process, about 4% of the total electron flux is lost, which leads to the formation of superoxide anions (the most potent of free radicals). This means that the flux of reactive oxygen species (ROS) is directly correlated with the intensity of energy metabolism: the faster the latter, the more ROS are produced in cells. Intense physical exercise is often associated with alterations and micro-trauma of the muscle membranes (*Kanter et al., 1988*): therefore, horses that are in most demand and give the best performance are those that generate more of these molecules, which are toxic when present in excessive quantities in cells where they are produced. These toxic ROS will quickly weaken cellular structures and impair their proper functioning. High performance horses that have been weakened will be more susceptible to have a "physiological accident" in the event of a peak of oxidative stress (heavy exertion, stressful situation, fatigue, etc. all of which are factors that may act alone or in combination).

In muscles, oxidative stress and inflammation disturb muscle fibre function by progressively weakening the fibre membrane and eventually leading to its partial or total rupture with release of intra-cytoplasmic enzymes such as creatine phosphokinase (CPK) and aspartate aminotransferase (AST) into the bloodstream ***(Fig. 1).*** Creatine phosphokinase (CPK) is an enzyme that promotes the storage and rapid release of energy by exchanging phosphates with ATP. It is particularly abundant in muscle tissue so it is therefore the

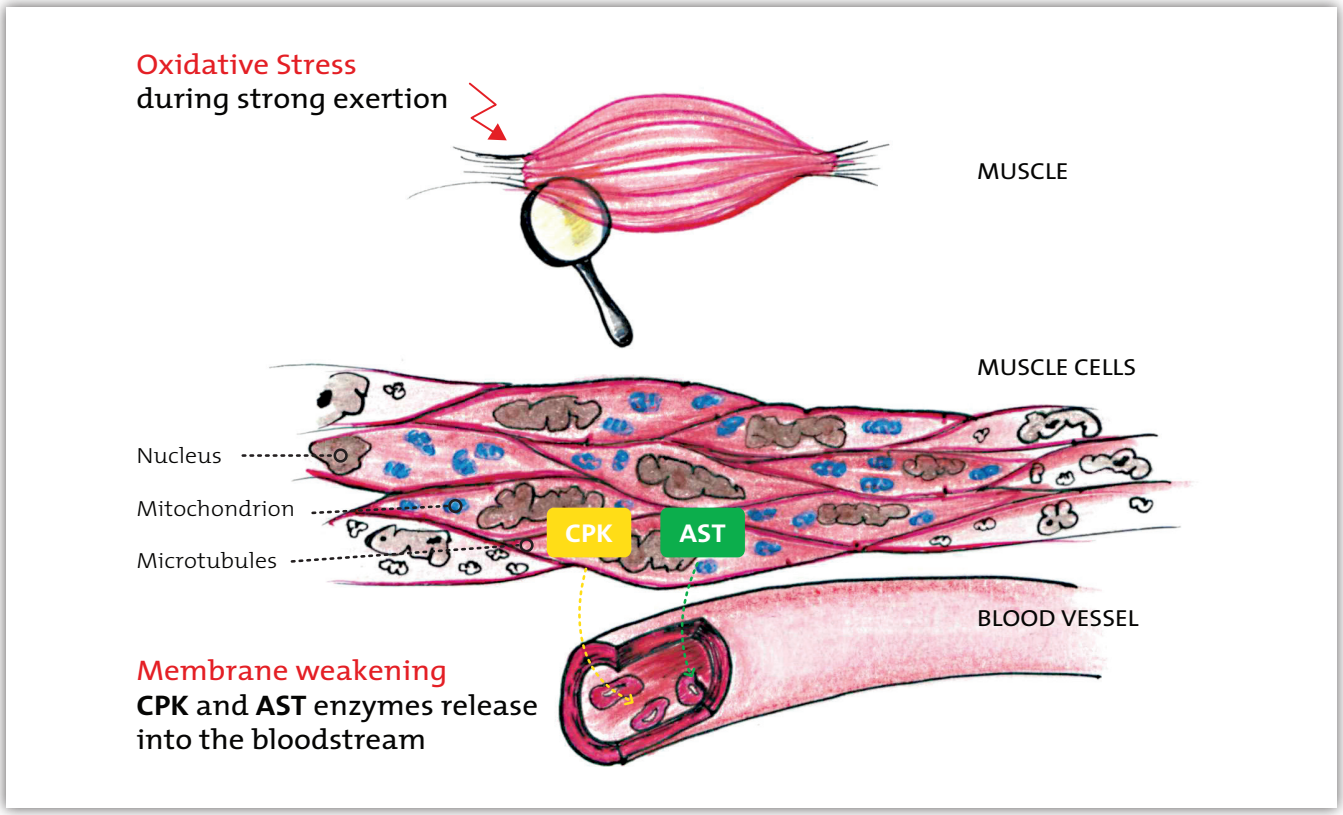


Figure 1: Diagram showing the release of muscle enzymes into the bloodstream after heavy exertion

main marker of cytolysis. The muscle enzyme activity after exertion (measured by the plasma concentration of these two enzymes) reflects the degree of membrane permeability induced by muscle function during exertion. The kinetics of CPK is very rapid with a peak at 3-4 h after exertion and a return to normal values within 24-72 hours. On the other hand, AST has slower kinetics with a delayed peak at 24 h after exertion and elimination in 7-10 days. The CPK and AST enzymes of race horses are therefore used as biomarkers by veterinarians to evaluate the integrity of muscle cells or the degree of "distress" of muscle cell membranes (in case of myositis after excessive muscle working and/or after insufficient warm-up, myopathies or muscle tearing).

1-2 Joint integrity

ANATOMICAL REVIEW OF JOINTS AND THEIR FUNCTIONING

Joints comprise at least two bone extremities linked by a capsule and ligaments. In horses, the joints are adapted to support a highly developed muscle mass (*cf. previous paragraph*) and to facilitate locomotion, and are particularly important to attain the level of performance that is specific to the equine species: instantaneous galloping speed frequently reaching 50 km/h (with a record of 70 km/h) or leaps as high as they are long (with a record height of 2.47 m!). The joints are therefore stabilised by a strong joint capsule, which links and solidly secures the bone extremities. This fibrous capsule is covered with a membrane known as the synovial membrane, which secretes a viscous liquid. The synovial fluid or synovium is present in the joint cavity formed by the membrane and the joint cartilage (at the bone extremity). Its most important functions are lubrication and nutrition of the joint tissue (*Bassleer et al., 1981*).

The role of the joint cartilage is essential since it minimises friction, efficiently conveys forces to the subchondral bone and maximises the contact surface under load. The cartilage changes shape under pressure but the periarticular structural elements and the bone bear most of the force. These properties are essentially a function of the organisation of the extracellular matrix. This matrix is mainly composed of proteoglycans, collagen fibres, fibronectin and anchorin. Proteoglycans are complex glycoproteins consisting of long chains of glycosaminoglycan (GAG) polysaccharides bound to a protein backbone. GAGs contribute to resistance and compression thanks to their ionic bonding with water. **GAGs are thus components of the cartilage matrix and their concentration in synovial fluid is generally considered to be a good indicator of cartilage catabolism.** Hence, a high synovium GAG content reflects a reduction in cartilage elasticity and in its capacity to withstand and convey forces efficiently ***(Fig. 2).***

MECHANISMS RESPONSIBLE FOR ALTERING JOINT FUNCTION FOLLOWING OXIDATIVE STRESS

Joint damage (or joint degeneration) is very common in horses (and more particularly in competition horses) and is one of the main causes of premature retirement of horses from racing (*Lejeune et al., 2006*): this observation confirms that the inner joint structure is fragile when it is exposed to violent and/or repetitive loading.

Repetitive exercise will rapidly cause chronic joint inflammation: the capsule thickens, the inflamed synovial membrane (*synovitis*) becomes hypertrophic and the cartilage begins to wear prematurely. This can generate such erosion that it can reach the underlying bone, which obviously makes the joint painful and impairs its function.

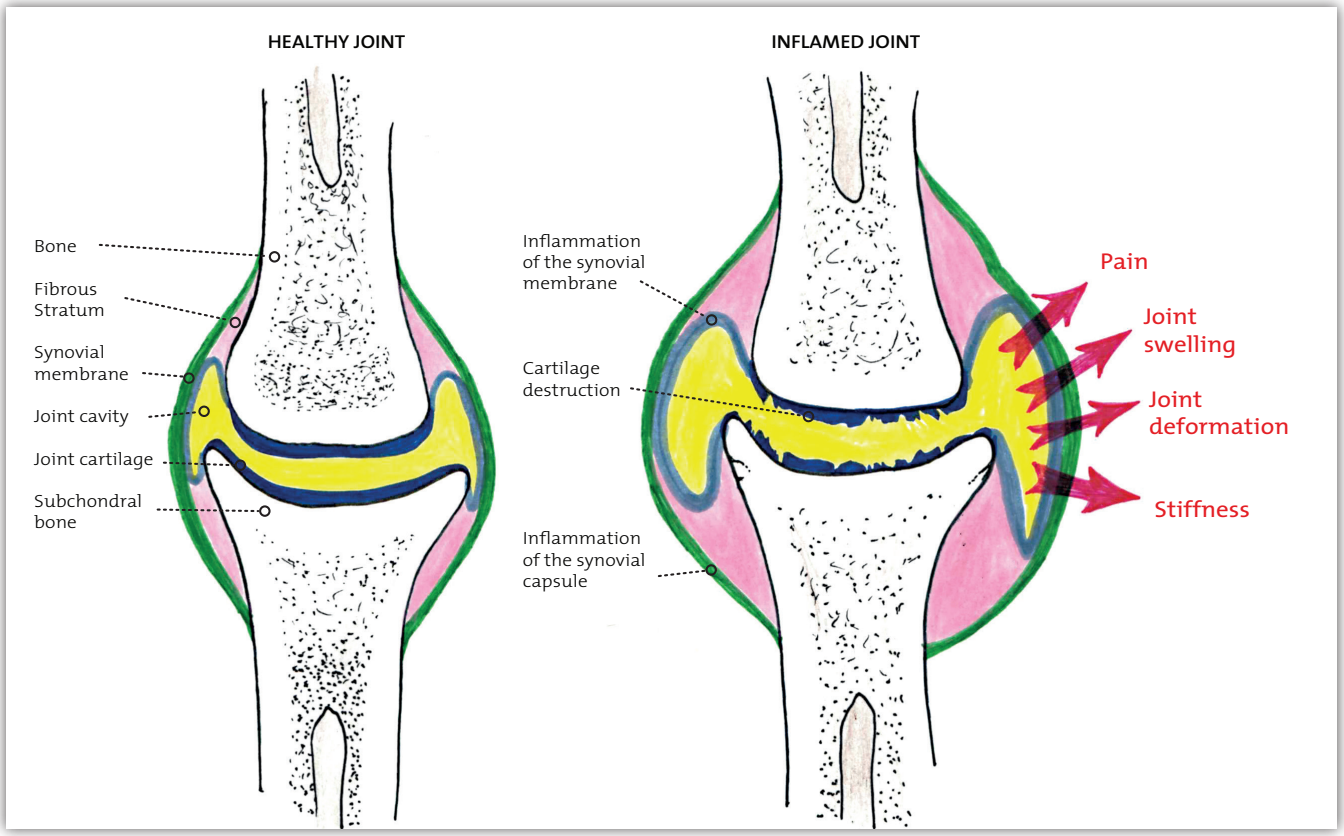


Figure 2: Diagram of a normal joint and an inflamed joint

As a result of chronic inflammation, the synovial membrane produces an excess of synovial fluid which accumulates in the joint cavity. The membrane becomes thicker and the joint swells, becoming even more painful. Over time, inflammation causes deep and permanent damage to the joint structural elements thereby causing cartilage fissures which gradually lead to destruction of the joint.

Equine degenerative osteoarthritis (DOA) is an inflammatory phenomenon that affects diarthrodial joints. This causes degradation of the cartilage and bone remodelling, and alters the synovial membrane. DOA involves all joint structural elements (cartilage, capsule, synovial membrane and bone) and is characterised by a disequilibrium between the processes of synthesis and degradation of the joint cartilage. This causes inflammation to set in and leads to the manifestation of clinical symptoms *(Lejeune et al., 2006)*. This soft tissue damage can affect several joint structural elements:

Soft tissue	Joint capsule and ligaments	Synovium of synovial fluid	Cartilage
Functions	Joint support	Lubrification and nutrition	Sliding and shock absorption
Possible alterations	Forced movements, stretching, ripping (*i.e. sprain*) or remodelling linked to a chronic process (arthrosis)	Acute phase inflammation (arthritis), chronic phase inflammation (arthrosis) or infection (septic arthritis)	Fissuring and degradation as a result of mechanical constraints or as part of inflammatory or degenerative processes (such as arthrosis)

An oxidising disequilibrium in joints manifests itself in the form of a rise in inflammation biomarkers such as prostaglandins (PGE_2) or nitric oxide (NO), and a higher concentration of cartilage matrix components (GAGs) in the synovial fluid (*Pearson et al., 2009*), which reveals the presence of cartilage damage that can lead to discomfort or even pain for the horse. The following paragraph describes the way markers are modified during intense physical exercise. These markers are valuable indicators of the health status of a horse's joints.

1-3 Effect of exercise on biomarkers in synovial fluid and plasma

Before exercising (-24 h), horses exhibit low GAG values in the synovial fluid and low blood plasma CPK/AST values (green circles), indicating an absence of damage in muscle and joint structural elements. The other points of the graphs below represent data at +1 h and +24 h after exercise: intense exercise rapidly increases the values of these biomarkers in horses, which reflects temporary cell and joint damage post-exercise (for more information on the protocol, please refer to paragraph 3–3, Trial 3) ***(Fig. 3).***

The values for the muscle enzymes CPK and AST are also at their lowest before exercise (-24 h) and increase gradually according to two distinct kinetic patterns: at +1 h for CPK, then at +24 h for AST (change kinetics for horses: green, orange then red area) ***(Fig. 3).***

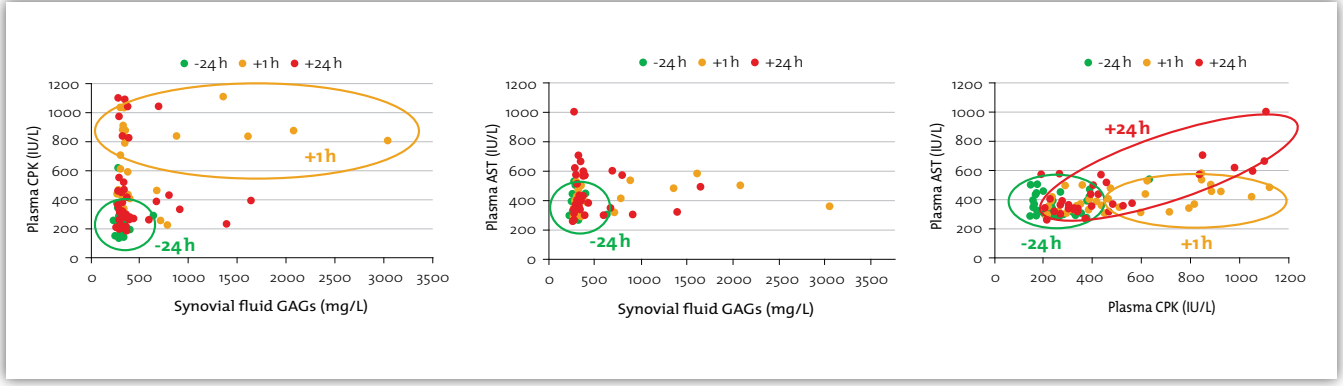


Figure 3: Effect of exercise on biomarkers in synovial fluid and plasma

BIOMARKERS OF SYNOVIAL FLUID

The effects of exercise on inflammation biomarkers (GAG, PGE_2, NO) and antioxidant defences (TAS, SOD) in the synovial fluid are shown in **Table 1.** Exercise provokes a significant increase in the GAG concentration 1h after exercise (-24 h vs +1 h: P = 0.05), the concentration at +24 h being intermediate between the values at -24 h and +1 h. A significant increase in the NO concentration is also observed at 1h after exertion (-24 h vs +1 h: P = 0.025) and +24 h after exercise, the increase tends to be significant (-24 h vs +24 h: P = 0.063). Exertion also increases the PGE_2 concentration and the SOD activity in a non-significant fashion.

Intense exercise therefore does indeed generate a temporary inflammation of the cartilage and damages its matrix.

PLASMA BIOMARKERS

The effects of exercise on plasma biomarkers of inflammation (PGE_2, NO) and of oxidative equilibrium (TBARS, TAS, SOD) or on biomarkers of muscle integrity (CPK, CRE, AST, ALP, GGT) are shown in **Table 1.**

Exertion significantly increases the activity of the enzyme CPK in plasma (-24 h vs +1 h: P < 0.001; -24 h vs +24 h: P = 0.001), AST (-24 h vs +24 h: P = 0.004; +1 h vs +24 h: P = 0.086) as well as the creatine level (-24 h vs +1 h: P = 0.002; +1 h vs +24 h: P = 0.001). It is worth noting that at 24 h, the CPK activity is intermediate between the values at -24 h and +1 h and that the creatine concentration has returned to the pre-exercise value (-24 h). The AST activity continues to increase between +1 h and +24 h. Exercise also increases the PGE_2 concentration and the ALP and GGT enzyme activity non-significantly, but does not seem to have any effect on plasma parameters such as TBARS, TAS and NO, or on the SOD activity.

These results confirm that exercise in this trial led to a degradation of the membrane of muscle fibres.

This study therefore confirms the effects of exercise on cartilage and the integrity of muscle fibres in horses. In the rest of this review, we wish to evaluate the benefit of **natural solutions aiming to improve the resistance of horses** by reducing the effects of exertion on both of these important performance elements: muscles and joints.

		-24 h	+1 h	+24 h	Probability
SYNOVIAL FLUID	GAG (mg/L)	298.5 (±12.5)^{A}	487.2 (±83.8)^{B}	409.9 (±53.1)^{AB}	0.061
	PGE_2 (ng/L)	249.4 (±30.6)	295.6 (±30.9)	260.9 (±27.7)	0.524
	NO (µM)	7.8 (±0.6)^{Aa}	22.5 (±5.2)^{B}	20.5 (±4.4)^{b}	0.019
	TAS (mM Trolox)	1.758 (±0.077)	1.667 (±0.085)	1.698 (±0.063)	0.688
	SOD (IU/mL)	79.5 (±5.5)	82.6 (±6.9)	84.8 (±6.4)	0.833
PLASMA	TBARS (µM)	0.644 (±0.075)	0.546 (±0.047)	0.529 (±0.042)	0.302
	PGE_2 (ng/L)	152.3 (±16.9)	275.4 (±64.7)	312.5 (±72.7)	0.114
	NO (µM)	48.0 (±3.5)	40.5 (±3.6)	50.6 (±3.6)	0.127
	TAS (mM Trolox)	0.915 (±0.050)	0.852 (±0.046)	0.887 (±0.052)	0.666
	SOD (IU/mL)	72.4 (±6.4)	74.1 (±4.4)	69.7 (±4.0)	0.830
	CPK (IU/L)	254.1 (±18.6)^{A}	537.6 (±46.2)^{B}	469.9 (±51.9)^{B}	<0.001
	CRE (µM)	115.1 (±5.8)^{A}	141.2 (±5.2)^{B}	114.9 (±4.2)^{A}	<0.001
	AST (IU/L)	354.6 (±12.6)^{A}	384.9 (±14.2)^{a}	443.6 (±27.7)^{Bb}	0.005
	ALP (IU/L)	169.9 (±7.8)	194.3 (±8.9)	175.4 (±8.9)	0.113
	GGT (IU/L)	25.9 (±1.4)	29.8 (±2.3)	28.8 (±2.4)	0.383

a, b: 0.05 <P < 0.1; A, B: P ≤0.05

Table 1: Plasma and synovium biomarkers analysed before (-24 h) and after (+1 h, +24 h) exercise (the values in parenthesis are the standard errors of the mean) (Barbé et al., JRE, 2017)

TAKE-HOME MESSAGES

- Striated muscles are sensitive to oxidative stress and inflammation that can lead to partial or total ruptures of their membranes. When it happens, muscular enzymes (CPK and AST) are released in the blood and they are used as biomarkers to evaluate the integrity of muscle cells.
- Synovial fluid lubricates and nourishes the joint tissues. Joint is composed of proteoglycans (GAG) that contribute to resistance and compression. The concentration of GAG is a good indicator of cartilage integrity.
- After exercise, as a result of chronic inflammation, the synovial membrane produces an excess of synovial fluid that can lead to joint damages with cartilage fissures. Biomarkers of inflammation (PGE_2, NO and GAG) reveal the presence of cartilage damage and are valuable indicators of the health status of a horse's joints.

2- Antioxidant solutions

2-1 MELOFEED

MELOFEED is a freeze-dried concentrate of melon juice, which is naturally rich in superoxide dismutase (SOD activity = min $2.6x10^6$ IU/kg) - the only enzyme able to dismutate the superoxide ion - and in catalase (SOD and CAT are two primary enzymes of the antioxidant defence system). MELOFEED is a unique source of natural SOD from plants, exclusively marketed and distributed by Lallemand for animal nutrition applications. The shelf life of the specific variety of melon used to produce MELOFEED is 4 times that of a traditional variety.

The original mechanism of action of MELOFEED is based on the stimulation of endogenous production of the 3 antioxidant enzymes (SOD, CAT and GPx) in several important tissues in animals administered MELOFEED.
This mechanism has been demonstrated in several organs (muscle, liver, heart, fat tissue and the reproductive tract) and in several animal species (rodents: rats/hamsters, laying hens, chickens, pigs), implying that there is a common process shared by all aerobic organisms (*Carillon et al., 2014, 2016*).

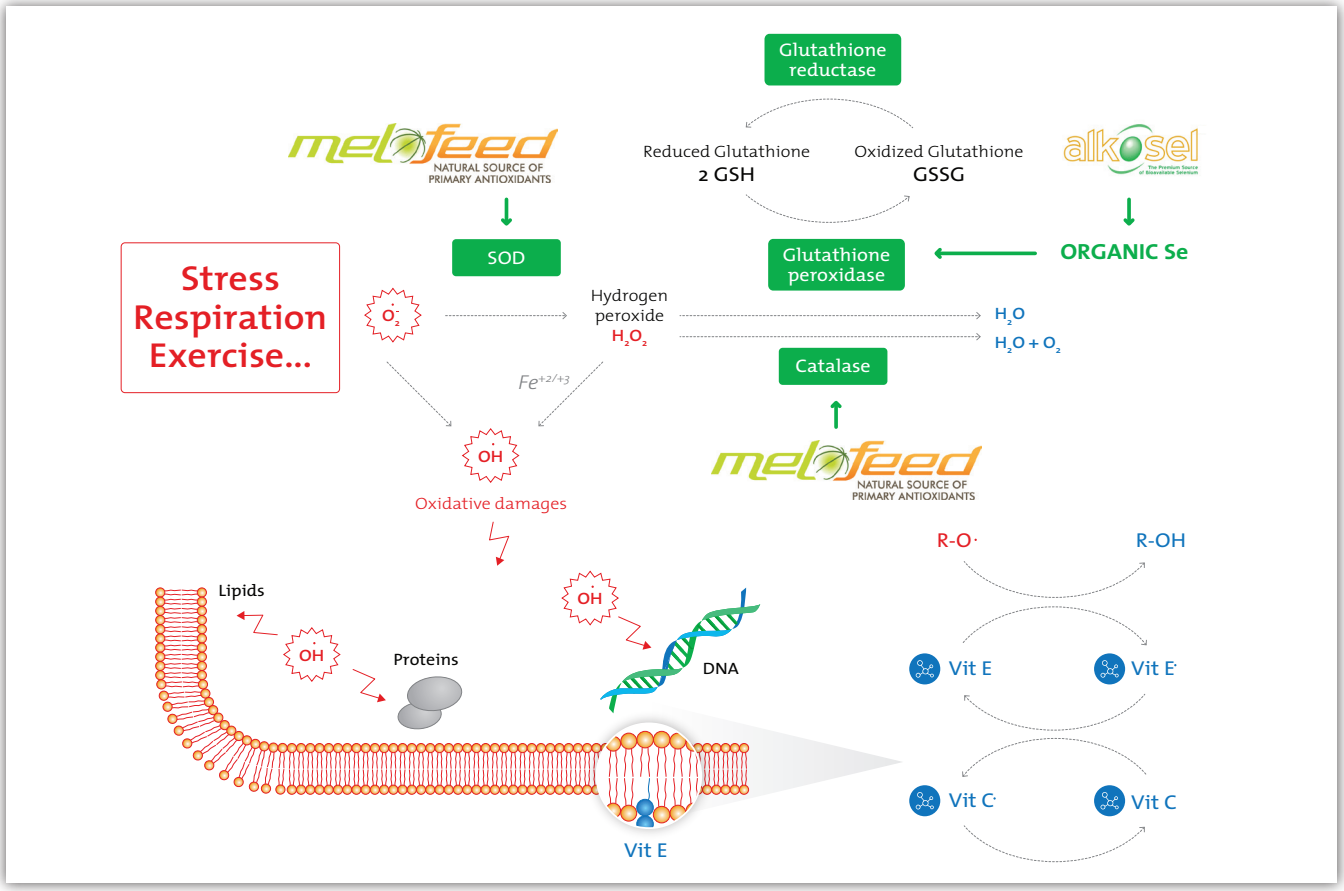


Figure 4: Stimulation of the first line of antioxidant defence with MELOFEED/ALKOSEL combination: MELOFEED stimulates the endogenous production of SOD, CAT and GPx; ALKOSEL supplies the GPx cofactor (Se)

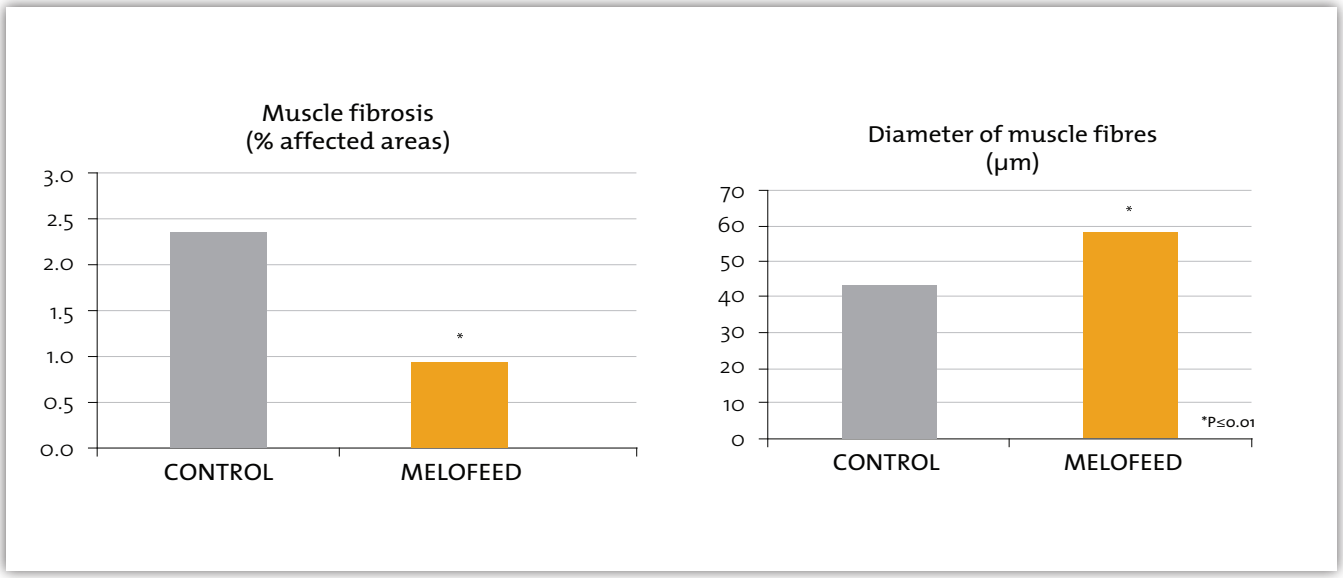


Figure 5: Beneficial effect of MELOFEED on muscle fibrosis and on the diameter of muscle fibres in a poultry model (Barbé et al., ESPN, 2017)

Organic selenium (ALKOSEL), a cofactor of glutathione peroxidase (GPx), enhances the effect of MELOFEED by acting in the subsequent step in which hydrogen peroxide (H_2O_2) is converted into water (H_2O) and oxygen (O_2). MELOFEED and ALKOSEL, which serve as a first line of antioxidant defence, therefore appear to act synergically to prevent the formation of reactive oxygen species and reduce oxidative stress ***(Fig. 4).***

MELOFEED can also protect muscle by reducing the risk of fibrosis and can stimulate muscle growth by increasing the diameter of muscle fibres ***(Fig. 5).***

Prostaglandins (PGE_2 in particular) are important mediators of inflammation and are produced by cyclo-oxygenases (COX) from arachidonic acid (AA), a Ω6 fatty acid with pro-inflammatory properties (*Kuehl & Egan, 1980*).

AA is itself produced from membrane phospholipids by phospholipase A_2 (PLA_2), which is induced by molecules such as organic peroxides (ROO°) and ROS (*Goldman et al., 1992*).

In addition, COX are also directly involved in ROS production (*Kim et al., 2008*) and their expression is increased in synovial fluid when joint disease is present (*Moulton, 1996; Siegle et al., 1998*). MELOFEED may therefore act at two different levels to reduce inflammation in the synovial fluid:

- 1) by limiting PLA_2 activation through reduction of secondary free radical production (organic peroxides) ***(Fig. 6, No. 1)***
- 2) by dismutating ROS products by COXs into less reactive molecules ***(Fig. 6, No. 2).***

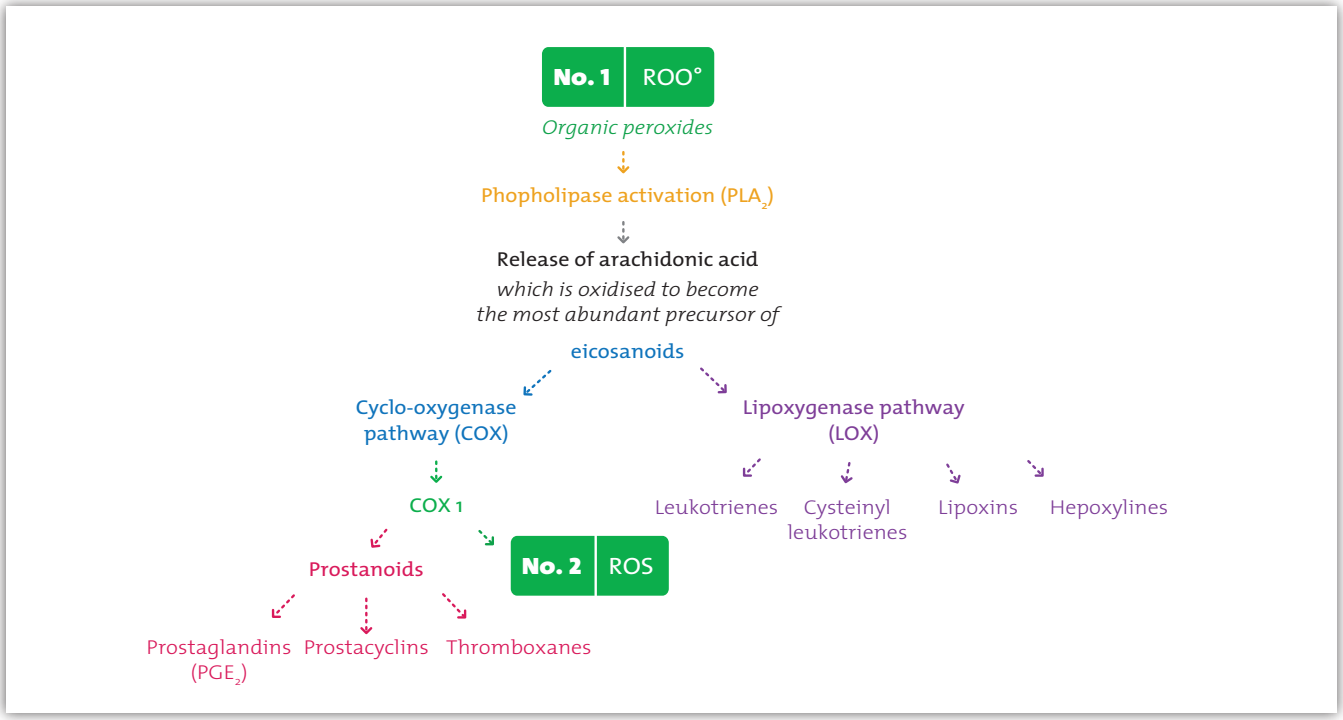


Figure 6: 2 sites of antioxidant action during the inflammatory process

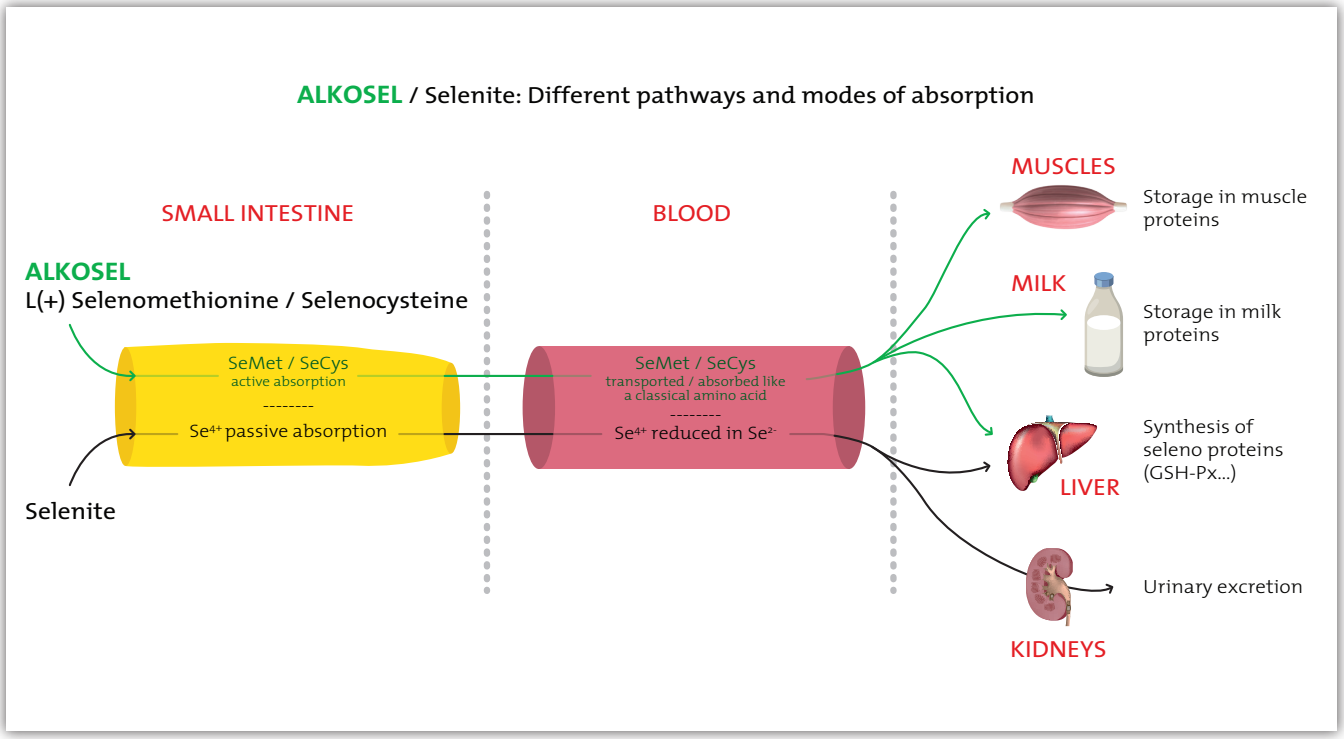


Figure 7: Passive absorption (inorganic source) and active absorption (organic source) of selenium

2-2 ALKOSEL

ALKOSEL is an inactive yeast that is rich in organic selenium (100% of the selenium occurs in organic form, the 98% guarantee allowing for the analytical variation in the measurements) that is essentially present in the form of selenomethionine (SeMet, minimum 63% of the organic forms of selenium) and selenocysteine (SeCys, around 17% of the organic forms of selenium in ALKOSEL). These forms can be assimilated by horses via the classic absorption path of sulphur amino acids and are far more bioavailable than mineral forms (*sodium selenite or selenate*) ***(Fig. 7).***

Selenium is an essential element in horse nutrition. Unfortunately, the natural concentration of selenium in forage and cereal is generally low. ALKOSEL thus provides quality selenium supplementation for optimum use. The concentration of selenium in horsehair after 6 months of supplementation with ALKOSEL demonstrates the excellent bioavailability of this source of organic selenium ***(Fig. 8).***

Selenium protects all cells and particularly muscle cells, thus contributing to preventing muscle degeneration (white muscle disease in foals, enzootic myodystrophy in adults, increased sensitivity to muscle stiffening and pain during or after exercise, myositis or rhabdomyolysis). Thanks to its anti-radical properties, selenium also protects membranes of the various cells of the immune system. *Arthur et al. (2003)* have shown that free radicals accumulate within neutrophils in which GPx activity is reduced, causing the death of these cells, which are necessary for proper function of the immune system. In addition, a deficiency in selenium or in the selenoproteins involved in the elimination of free radicals can lead to malfunction of the inflammatory response and to an aggravation of infection: *Streeter et al. (2012)* have shown that hospitalised adult horses have very low levels of selenium.

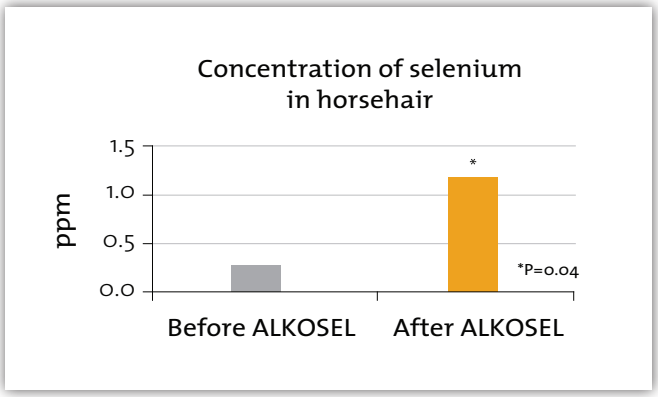


Figure 8: Se concentration in horsehair after 6 months of ALKOSEL supplementation (N = 5) per day/horse (Marsh, 2013)

Finally, selenium plays a crucial role in fertility and the reproductive process as well as in the transfer of immunity to the foal. *Bertelsmann et al. (2010)* have shown that selenium affects sperm quality and male fertility since some of the selenoproteins are involved in spermatogenesis and sperm maturation. *Weiss and Hogan (2005)* have emphasised the effect of ALKOSEL supplementation administered to the mare, to raise the selenium concentration in colostrum and newborn serum.

TAKE-HOME MESSAGES

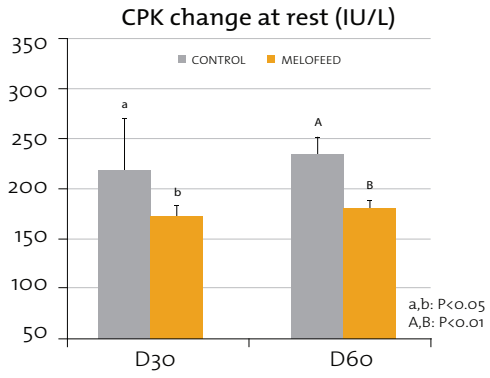
- MELOFEED, freeze-dried concentrate of melon juice, stimulates endogenous primary antioxidant enzymes expression (SOD, catalase and GPx). This mode of action increases the levels of primary antioxidants to keep an optimal oxidative balance, even during stressful physiological or environmental periods.
- MELOFEED can also protect muscles by reducing the risk of fibrosis and can stimulate muscle growth by increasing the diameter of muscle fibers.
- ALKOSEL is an inactive yeast, rich in organic selenium, essentially present in the form of selenomethionine (SeMet, minimum 63% of the organic forms of selenium) and selenocysteine (SeCys, around 17% of the organic forms of selenium in ALKOSEL).
- Horses assimilate ALKOSEL via the classic absorption path of sulphur amino acids, and are far more bioavailable than mineral forms (sodium selenite or selenate).
- Organic selenium protects cells including muscle cells, thus contributing to prevent muscle degeneration, and is involved in the elimination of free radicals that can lead to malfunction of inflammatory response and to an aggravation of infection.
- MELOFEED and ALKOSEL stimulate the first line of antioxidant defence, they work synergistically to prevent the formation in excess of reactive oxygen species and to reduce oxidative stress.

3- Equine applications

3-1 Trial 1: Resistance of muscle membranes in trotters (France, 2010)

In 2010, a double blind study (*Notin et al., 2010*) was conducted (neither the trainer nor the investigator knew what batches A and B contained) in order to demonstrate and quantify the effects of MELOFEED on the physiological parameters of exertion and on the markers of oxidative stress in a population of race horses. 24 French Trotters, all of them in training, in a period of competition or preparation for qualification, were matched pair-wise by age, sex and training stage. Two groups were then formed at random: one group receiving MELOFEED (200 mg/horse/day, n = 12) and one group receiving control feed (n = 12) for 60 days. Blood biomarkers were analysed half way through (at day 30) and at the end of supplementation (at day 60).

The muscle enzyme activity was measured with **CPK (creatine phosphokinase).** Antioxidant defences were evaluated by analysing the **SOD activity in red blood cells** and using the **KRL test.** This test, based on haemolysis induced by attack by free radicals, evaluates the antiradical resistance of the blood and red blood cells. Intra- and extra-cellular antioxidant defences contribute to the maintenance of the membrane integrity and the proper function of cells exposed to damage by radicals, which results in red blood cell haemolysis. This biological test allows dynamic measurement of the general antiradical defence potential in the circulation and within cells in horses. The resistance of blood and red blood cells to attack by free radicals is expressed by the time taken for 50% of red blood cells to lyse (time to 50% haemolysis, T1/2 in minutes). The antiradical efficiency of plasma is measured by the difference between resistance measured for total blood and the resistance measured for red blood cells.



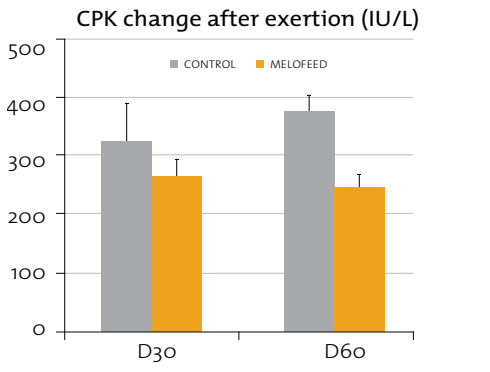


Figure 9a: Effect of MELOFEED on the muscle enzyme activity (CPK) in trotters

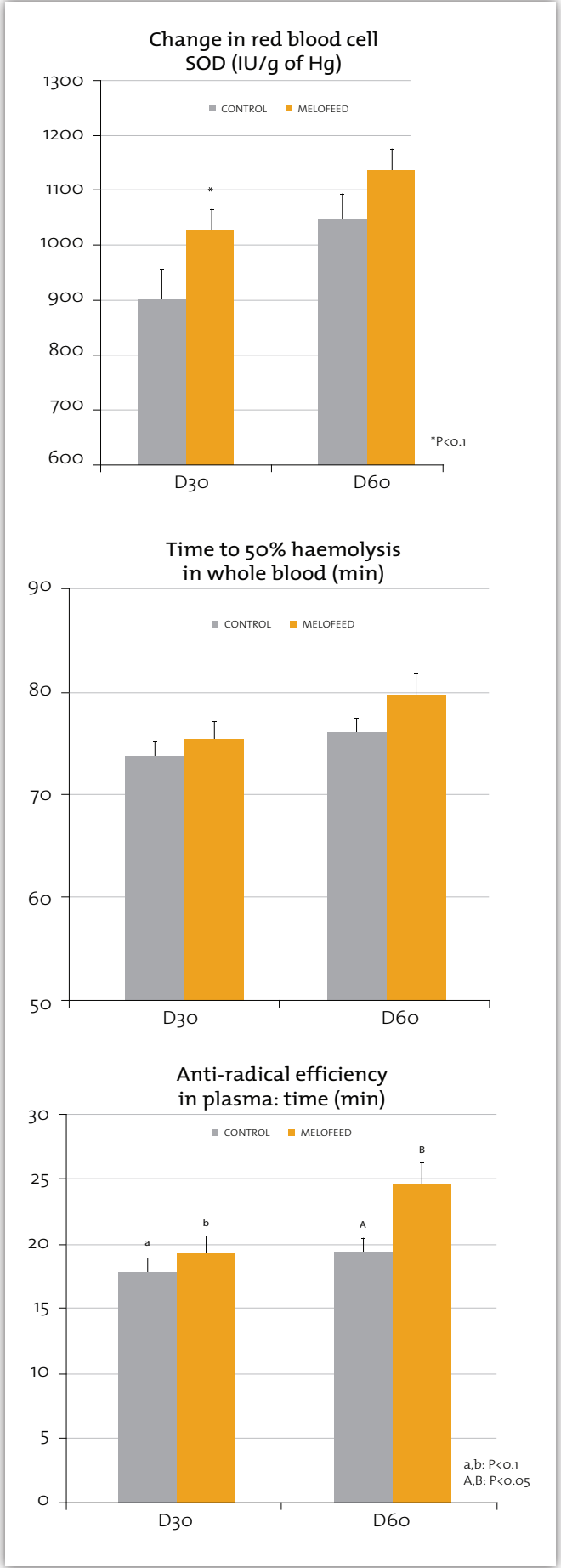


Figure 9b: Effect of MELOFEED on antioxidant defenses (SOD activity, results of KRL test for whole blood and plasma) in trotters

At Do, the CPK enzyme concentrations at rest are not significantly different between the two groups (P = 0.14). The CPK activity at rest appears to be significantly lower in the MELOFEED group at D30 (P = 0.039) and D60 (P = 0.007). At D30 and D60, the post-effort enzyme concentrations were higher in the control group than in the MELOFEED group in a quasi-significant manner (respectively P = 0.085 and P = 0.11) ***(Fig. 9a).*** The levels of red blood cell SOD were higher in the MELOFEED group than in the control group in a quasi-significant manner at D30 (P = 0.079) and numerically higher at D60 (P = 0.17) ***(Fig. 9b).***

At D60, a strong trend was observed: the time to 50% haemolysis of whole blood was higher in the MELOFEED group than in the control group in a quasi-significant manner (P = 0.13). This observation is all the more pronounced for plasma radical efficiency which shows a significant advantage for MELOFEED at D30 (P < 0.1) and D60 (P < 0.05) compared to the control group ***(Fig. 9b).***

This trial showed that supplementation with MELOFEED in race horses offers benefits in terms of muscle cell membrane protection, stimulation of the antioxidant defence system and antiradical potential of blood. Therefore, MELOFEED helps prevent the increase of muscle membrane permeability linked to intense training whilst stimulating the antioxidant defence potential.

3-2 Trial 2: Protection of muscle membranes in race horses (France, 2014)

In 2014, another study (*Barbé et al., 2014*) was conducted in order to analyse the effect of a combined antioxidant supplementation (MELOFEED: 200 mg/horse/day, ALKOSEL: 500 mg/horse/day, vitaminE: 1.25 mg/horse/day) on the muscle enzymes CPK and AST, which are markers of membrane integrity of muscle cells, in a context of rhabdomyolysis (muscle inflammation: paroxystic myoglobinuria, or myositis though the latter term is a misnomer).

A decrease in the activity of these enzymes in the blood indicated that cell membranes were more efficiently protected and that muscle integrity was therefore improved.

37 horses were assigned to three different groups:

- 1) The red group (n = 8): resting horses (with myositis)
- 2) The orange group (n = 11): working horses displaying high initial CPK/AST values
- 3) The green group (n = 18): working horses with a history of myositis but not giving high CPK/AST values at the beginning of antioxidant supplementation

The duration of antioxidant supplementation was 31 weeks, 10 weeks and 15 weeks for the green, orange and red groups, respectively ***(Fig. 10).*** The blood enzyme activity of CPK (*creatine phosphokinase*) and AST (*aspartate aminotransferase*) was measured weekly. The graphs below show the mean change in blood concentration of CPK and AST (IU/L) for each group of horses over time (in weeks of antioxidant supplementation). The first value (0) in grey represents the mean value before antioxidant supplementation. The horizontal black lines represents the critical reference values, set at 370 IU/L for CPK and 460 IU/L for AST. The horses from the orange and red group yielded high initial CPK and AST values (the horses of the red group were at rest). The CPK values were maintained

Figure 10: Effect of combined antioxidant supplementation on CPK and AST in red, orange and green groups

below the critical value of 370 IU/L as of the first week of antioxidant supplementation. The AST values were also reduced as time went on with final values below 460 IU/L at 10 weeks of antioxidant supplementation for the red group and as of the third week of antioxidant supplementation for the orange group. These results also confirm the slower kinetics of AST compared to CPK ***(Fig. 10).*** The horses from the green group showed initial CPK/AST values that were lower than the critical values. Long-term MELOFEED/ALKOSEL/ vitaminE supplementation (31 weeks) allows these values to be maintained at optimum levels ***(Fig. 10).***

In conclusion, for horses with high initial values, this antioxidant supplementation helps reduce the values of the blood CPK and AST parameters as early as the first week of supplementation. This antioxidant combination also allows long-term maintenance of optimum values of these parameters for horses with initial values that are lower than the critical values.

MELOFEED and ALKOSEL therefore have a beneficial effect on the restoration and maintenance of the membrane integrity of horse muscle cells, and hence on resistance to strain.

3-3 Trial 3: Maintenance of the muscle and joint integrity in leisure horses (Canada, 2017)

In 2015, a joint fatigue model was developed in Canada in order to investigate the significance of oxidative stress and inflammation produced during intense physical exercise, and to evaluate the benefits of the primary antioxidant superoxide dismutase (SOD) afforded by MELOFEED (*Barbé et al., JRE 2017*). SOD is the only enzyme capable of transforming (dismutating) the superoxide anion into hydrogen peroxide, which preserves the oxidation-reduction equilibrium and therefore maintains the integrity of cellular structures.

This study was designed to evaluate the impact of intense exercise and MELOFEED supplementation on various synovial fluid and plasma biomarkers, which are indicators of oxidative equilibrium, inflammation and cell membrane integrity.

12 horses were included in the study. 4 horses received control feed for 23 days (control group, C), 4 horses received MELOFEED supplementation (group MELOFEED, M: 1g/horse/day) for 23 days and 4 horses first received the MELOFEED supplementation for 23 days followed by the control feed for 23 days after a wash-out period of 21 days. As the periods were considered to be independent, the results were therefore analysed for 8 horses per group. Over each period, each horse exercised twice (on the first and last day of supplementation) ***(Fig. 11).*** The exercise consisted in 4 full-gallop races in the mud over a distance of 800 metres with a 4-minute walk to rest between each race, the effort being regulated according to heart rate.

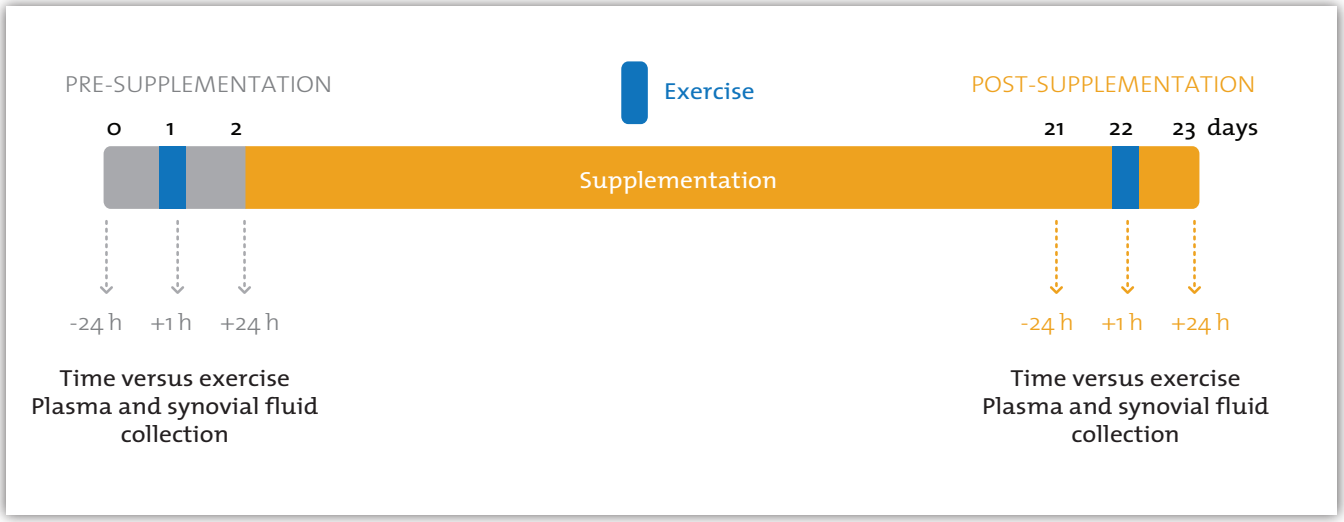


Figure 11: Experimental protocol

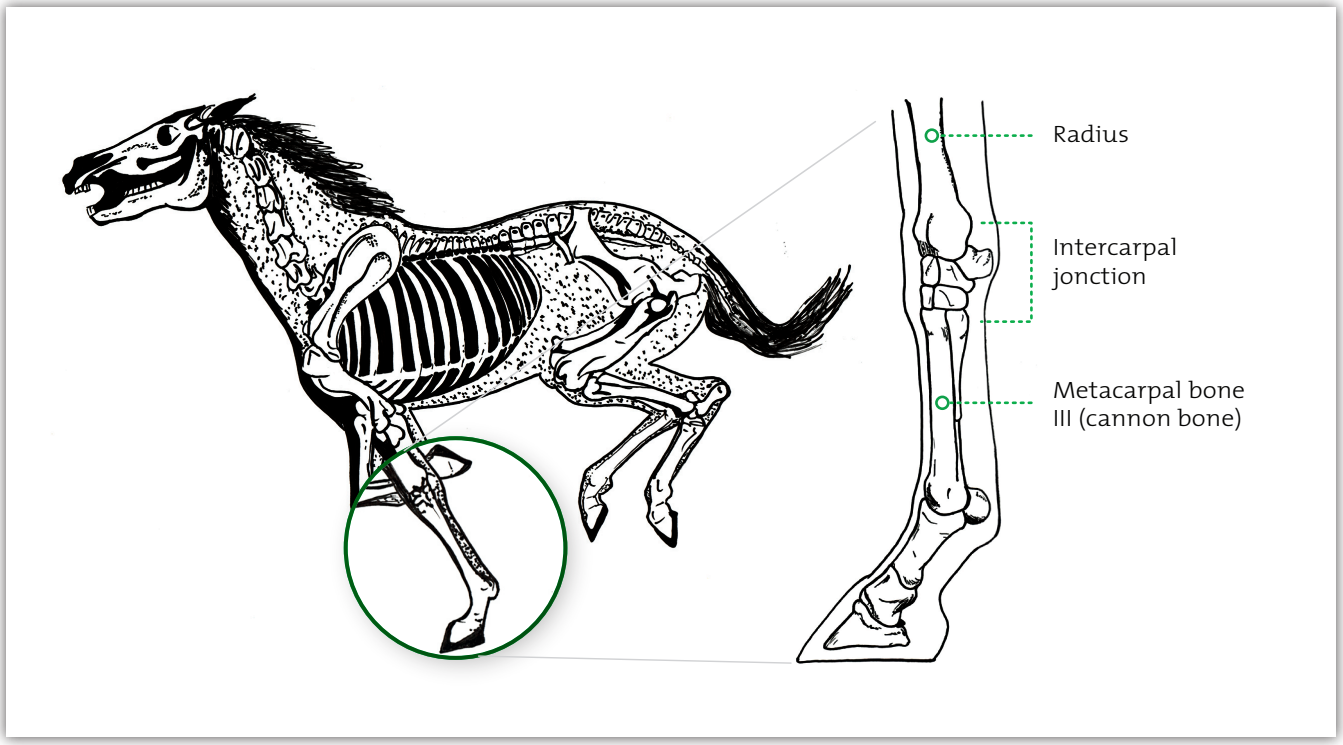


Figure 12: Sampling location of liquid fluid in intercarpal jonction

Time	Group	GAG (mg/L)	PGE_2 (ng/L)	NO (µM)	TAS (mM of Trolox)	SOD (IU/mL)
-24 h	Control (C)	348.2 (±36.8)	380.5 (±65.9)	8.8 (±1.4)	1.621 (±0.035)	68.9 (±8.6)
	MELOFEED (M)	289.0 (±36.9)	281.4 (±66.2)	6.7 (±1.3)	1.639 (±0.036)	64.2 (±8.7)
+1 h	Control (C)	817.6 (±230.5)	397.8 (±62.0)	43.7 (±13.1)	1.395 (±0.106)	56.4 (±11.9)
	MELOFEED (M)	307.8 (±230.5)	233.2 (±61.1)	10.9 (±13.1)	1.629 (±0.106)	85.8 (±12.0)
+24 h	Control (C)	305.1 (±16.4)	306.8 (±54.4)	13.0 (±3.1)	1.648 (±0.043)	65.2 (±9.8)
	MELOFEED (M)	316.9 (±20.0)	196.7 (±54.3)	11.7 (±3.7)	1.645 (±0.042)	82.0 (±10.0)
	Probability	0.121 (C > M)	0.017 (C > M)	0.082 (C > M)	0.169 (C < M)	0.105 (C < M)

Table 2: Synovial fluid biomarkers in both groups (control, MELOFEED) analysed before (-24 h) and after (+1 h, +24 h) exercise (post-supplementation data analysis; the values in parenthesis are the standard errors of the mean)

This experimental protocol was chosen as a model for the induction of moderate oxidative stress, associated with moderate inflammation in muscles (*Liburt et al., 2010; Powers et al., 2011a*) and joints (*Reed et al., 2012; Welsh et al., 2013*).

The synovial fluid from the intercarpal jonction ***(Fig. 12)*** and blood from the jugular vein were collected according to the method of *Pearson et al. (2009)* at 3 time points: before exercise (-24 h) and at 1h and 24 h after exercise ***(Fig. 11).***

Several blood and joint biomarkers for oxidative stress and inflammation were then analysed: the inflammatory status was evaluated according to the PGE_2 (prostaglandin E_2) and **NO** (nitric oxide) content and the antioxidant status was evaluated according to the **SOD** (superoxide dismutase) activity and the **TAS** (total antioxidant status). In addition, the concentration of glycosaminoglycans **(GAG)** in synovial fluid was analysed after the method of *Chandrasekhar et al. (1987),* and the plasma levels of **TBARS** (thiobarbituric acid reactive substances, a marker of lipid peroxidation) were determined at the indicated time points. The damage to muscle membranes during exercise was evaluated before exercise (-24 h) and at 1h and 24 h after exercise by analysing the plasma activity of several muscle enzymes: creatine phosphokinase **(CPK)**, aspartate aminotransferase **(AST)**, alkaline phosphatase **(ALP)**, gamma-glutamyl transferase **(GGT)**, and creatine concentration **(CRE)**.

BIOMARKERS OF SYNOVIAL FLUID

The effects of MELOFEED supplementation on biomarkers of inflammation (GAG, PGE_2, NO) and antioxidant defences (TAS, SOD) in the synovial fluid are shown in **Table 2**. This supplementation with primary antioxidant induces a significant reduction in the PGE_2 concentration (C: 361.7 ng/L; M: 237.1 ng/L; P = 0.017) and a reduction following a trend for NO concentrations (C: 21.8 µM; M: 9.8 µM; P = 0.082) and in GAG (C: 490.3 mg/L; M: 304.6 mg/L; P = 0.121). **MELOFEED therefore reduces the risk of inflammation and of alteration of the cartilage matrix.** MELOFEED supplementation induces an increase in SOD activity (C: 63.5 IU/mL; M: 77.3 IU/mL; P = 0.105) and in the TAS parameter (C: 1.555 mM of Trolox; M: 1.638 mM of Trolox; P = 0.169).

MELOFEED thus participates in the stimulation of antioxidant defences of synovial fluid.

Correlation analysis indicates a positive correlation between the GAG and NO levels (R^2 = 0.95, P < 0.001), a negative correlation between the GAG and TAS levels (R^2 = 0.69, P < 0.001) and a negative correlation between the NO and TAS levels (R^2 = 0.74, P < 0.001). After intense exercise, MELOFEED reduces the proportion of horses with cartilage lesions (*i.e.* with synovial fluid GAG concentrations above 400 µg/mL **Table 3**). While the population of horses affected remains the same in the control group (37.5%) or even gets worse: after exercise, 2 horses had GAG levels above 1000 µg/mL in the joints, there were no more horses with GAG levels > 400 µg/mL (or > 1000 µg/mL) in the MELOFEED group.

Thus MELOFEED was able to reduce the harmful effects of intense exertion on cartilage damage by contributing to the maintenance of good integrity, particularly in fragile horses, while helping them to recover physically from previous damage.

PLASMA BIOMARKERS

The effects of MELOFEED supplementation on biomarkers of inflammation (PGE2, NO) and of the oxidative equilibrium (TBARS, TAS, SOD) in plasma as well as on muscle damage (CPK, CRE, AST, ALP, GGT) are shown in **Table 4.**

Before → After supplementation	Percentage of CONTROL HORSES	Percentage of MELOFEED HORSES
Cartilage lesions [GAG] > 400 µg/mL	37.5 % → 37.5 %	50 % → 0 %
Major cartilage alteration [GAG] > 1000 µg/mL	0 % → 25 %	25 % → 0 %

Table 3: Evolution of the population of horses with joint damage after intense exercise on the first day of the trial and after 1 month of supplementation with MELOFEED

Time	Group	TBARS (µM)	PGE_2 (ng/L)	NO (µM)	TAS (mM of Trolox)	SOD (IU/mL)
-24 h	Control (C)	0.794 (±0.151)	279.1 (±38.8)	58.7 (±7.3)	1.103 (±0.028)	59.0 (±12.0)
	MELOFEED (M)	0.476 (±0.145)	180.1 (±33.3)	50.0 (±6.6)	1.078 (±0.026)	81.8 (±11.2)
+1 h	Control (C)	0.492 (±0.093)	338.9 (±80.5)	49.0 (±7.5)	0.986 (±0.025)	74.7 (±8.9)
	MELOFEED (M)	0.529 (±0.093)	185.0 (±79.7)	52.1 (±7.5)	1.049 (±0.025)	62.4 (±8.9)
+24 h	Control (C)	0.571 (±0.099)	378.7 (±85.4)	58.6 (±7.4)	1.001 (±0.071)	82.4 (±5.7)
	MELOFEED (M)	0.452 (±0.099)	154.2 (±84.2)	52.8 (±7.2)	1.106 (±0.071)	72.0 (±5.6)
	Probability	0.162 (C > M)	0.012 (C > M)	0.536 (C > M)	0.227 (C < M)	0.997 (C = M)

Time	Group	CPK (IU/L)	CRE (µM)	AST (IU/L)	ALP (IU/L)	GGT (IU/L)
-24 h	Control (C)	328.5 (±51.6)	123.2 (±4.2)	345.7 (±20.0)	128.4 (±6.6)	24.5 (±2.0)
	MELOFEED (M)	368.5 (±47.9)	120.3 (±4.3)	402.9 (±21.9)	175.8 (±6.6)	25.6 (±2.0)
+1 h	Control (C)	569.1 (±72.6)	129.9 (±6.5)	394.9 (±29.1)	179.3 (±9.6)	34.5 (±6.6)
	MELOFEED (M)	392.9 (±68.7)	130.4 (±6.7)	385.6 (±31.3)	171.6 (±10.1)	24.7 (±7.0)
+24 h	Control (C)	629.1 (±88.9)	122.8 (±3.6)	456.2 (±56.4)	172.5 (±7.8)	34.2 (±6.3)
	MELOFEED (M)	304.9 (±94.1)	125.6 (±4.0)	361.1 (±58.9)	178.1 (±8.4)	21.9 (±6.7)
	Probability	0.013 (C > M)	0.972 (C = M)	0.634 (C > M)	0.670 (C > M)	0.133 (C > M)

Table 4: Plasma biomarkers in both groups (control, MELOFEED) analysed before (-24 h) and after (+1 h, +24 h) exercise (the values in parenthesis are the standard errors of the mean)

This supplementation with primary antioxidant induces a significant reduction in the CPK enzyme activity (C: 508.9 IU/L; M: 355.4 IU/L; P = 0.013) and in the PGE_2 concentration (C: 332.2 ng/L; M: 173.1 ng/L; P = 0.012).

This supplementation also has a non-significant beneficial effect on the TBARS concentration (C: 0.619 µM; M: 0.486 µM) and NO concentration (C: 55.4 µM; M: 51.7 µM), on antioxidant defences (TAS = C: 1.030mM of Trolox; M: 1.078 mM of Trolox) and on the other biomarkers of muscle integrity (AST = C: 398.9 IU/L; M: 383.2 IU/L - ALP = C: 178.1 IU/L; M: 175.2 IU/L - GGT = C:31.1 IU/L; M: 24.1 IU/L).

MELOFEED thus helps to prevent muscle lesions occurring in horses during intense exercise.

In this context, the model for muscle and joint oxidative stress proposed in this study proves to be a useful tool for following the kinetics of various markers of inflammation and oxidative stress, and for validating efficient solutions that might prevent the occurrence of irreversible muscle and joint damage. Indeed, the results of this study (described in paragraph 1–3) show the effect of intense exercise, such as cross-country exercise, on joint and muscle health and allow validation of the experimental protocol: **challenging horses increases synovial fluid inflammation (NO), causes degradation of cartilage structures (GAG) and weakens muscle membranes (CPK, CRE, AST).**

These observations confirm the results of other studies which have shown an increase in the markers of inflammation and oxidative stress in the blood and synovial fluid (*Fernandez-Moreno et al., 2011; Fuller et al., 2001; Powers et al., 2011b; Shang et al., 2009*), as well as an increase in the GAG plasma levels (*Calatroni et al., 2008*), following repeated physical exercise. In addition, excessive production of reactive oxygen species (ROS) in muscles and joints contribute directly to the inflammation of these tissues (*Powers et al., 2011b; Sies, 1997*). While the consequences of oxidative stress and the inflammatory process on the development of pathologies of muscle tissue and joint structures in horses are well established and proven, as they have been in this study, it is worth remembering that moderate inflammation is part of the natural physiological process of repair of damaged tissue. Thus the maintenance of a proper oxidation/reduction equilibrium, for instance by preventing the production of excessive ROS which lead to long-term tissue damage, is an approach worth exploring.

In this respect, MELOFEED appears to be an efficient means of defence against oxidative stress after intense physical exertion. This study confirms the observations on the maintenance of muscle integrity and provides new information on the reduction of inflammation (PGE_2, NO) and on the stimulation of SOD enzyme activity in synovial fluid, associated with the maintenance of cartilage integrity (GAG) following supplementation with MELOFEED.

TAKE-HOME MESSAGES

- MELOFEED used to improve the resistance of muscle membrane in trotters, shows benefits in terms of muscle cell membrane protection, stimulation of antioxidant defence system and induction of the antiradical potential of blood. Therefore, MELOFEED helps to prevent the increase of muscle membrane permeability linked to intense training whilst stimulating the antioxidant defence potential.
- MELOFEED and ALKOSEL used to improve the protection of muscle membranes, in race horses, show beneficial effects on the restoration and the maintenance of the membrane integrity of horse muscle cells, and hence on the resistance to strain.
- MELOFEED, used to improve the maintenance of muscle and joint integrity in leisure horses, shows, thanks to the measurement of biomarkers of synovial fluid, that its supplementation is able to reduce the harmful effects of intense exertion on joint damage by contributing to the maintenance of good joint integrity, particularly in fragile horses, while helping them to recover physically from previous damage. Thanks to the measurement of plasma biomarkers, it is also confirmed that MELOFEED helps to prevent muscle lesions occurring to horses during intense exercise.

General conclusion and prospects

The fragility of muscle and joint membranes in horses is a reality. Intense training of race horses is an important source of oxidative stress linked to the excessive production of ROS during the cellular respiration process. Oxidative stress has a negative effect on resistance during training because it weakens cell membranes and prevents optimal cell function. Joints and muscles are key physiological compartments. It is therefore crucial to protect them in order to preserve the performance potential of athlete horses.

The antioxidant solutions MELOFEED and ALKOSEL presented and tested in the different trials constitute an efficient means of defence against oxidative stress following intense physical exertion. An innovative approach to combat oxidative stress is to act at the level of the first antioxidant line of defence by stimulating the antioxidant enzymes: superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). In an animal model, the ingestion of SOD in the form of MELOFEED can induce the endogenous expression of these three enzymes. Thus, MELOFEED has a strong antioxidant potential thanks to the stimulation of the entire first line of antioxidant defence. As a GPx cofactor, organic selenium (ALKOSEL) enhances the effect of MELOFEED by acting during the phase of hydrogen peroxide elimination. Thus, the combination of MELOFEED and ALKOSEL, which act in a complementary fashion on the first line of antioxidant defence, is of crucial importance in preventing the formation of excessive ROS and controlling oxidative stress, and consequently **preserving the performance capital of our horses.**

These studies open new prospects in primary antioxidant supplementation with a view to maintain the muscle and joint integrity of athlete horses, horses suffering from joint pathologies and/or ageing horses but may also be applicable to other species such as the canine species.

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