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A new method to objectively evaluate animal welfare in intensive pig farming

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CHAPTER 1

Foreword

1.1 Oxidative stress

The term “oxidative stress” describes a biological condition in which the generation of oxidizing species is higher than the capacity of the organism to detoxify them. Such oxidizing species are mainly the result of the metabolism of oxygen. Using the reactive potential of oxygen, multicellular organisms have acquired a much higher efficiency in energy metabolism. The oxygen-driven reactions bear the potential of forming intermediate species able to damage cells and tissues. During evolution a number of detoxifying mechanisms evolved aimed at reducing the damaging capacity of reactive oxygen species (ROS) or repairing ROS-related damage. The concept “oxidative stress” applies only if these protective or repair systems are overwhelmed. Oxidative stress may result from enhanced ROS formation, or from the malfunction of the scavenging systems. As a consequence of this, and due to the fact that oxidants have multiple generation sites and the detoxifying systems involve several dozen of enzymes and compounds, oxidative stress is a complex phenomenon, difficult to assess and, therefore, hard to measure (Grune and Berger, 2007).

1.1.1. ROS

The threat of uncontrolled oxidation of biomolecules largely comes from the so-called reactive oxygen species (ROS). The term ROS is used to cover both the free radical and non-radical oxidants; a free radical contains at least one unpaired electron in the shells around the atomic nucleus and are capable of independent existence. The radical group includes species such as hydroxyl radical ($\text{OH}\cdot$), nitric oxide ($\text{NO}\cdot$) and superoxide ($\text{O}_2^{\cdot-}$). Compounds can also be highly reactive without being radicals. Such non-radical oxidants include peroxynitrite (ONOO^-), hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl) (Tab. 1).

From a chemist’s perspective, the formation and reactivity of ROS is rather intriguing. Molecular oxygen in air is triplet dioxygen; although it is a diradical, and requires a total of four electrons for reduction to water, it reacts slowly with many biomolecules (e.g. DNA, proteins and membranes). If reduced, however, O_2 is turned into highly aggressive ROS, which are more reduced yet, at the same time, more oxidizing than triplet oxygen. One-electron reduction results in the superoxide anion, two-electron reduction in hydrogen peroxide and three-electron reduction in the highly destructive hydroxyl radical.

Name	Symbol
Oxygen radicals	
Oxygen (bi-radical)	$O_2^{\cdot\cdot}$
Superoxide ion	$O_2^{\cdot-}$
Hydroxyl	OH^{\cdot}
Peroxyl	ROO^{\cdot}
Alkoxy	RO^{\cdot}
Nitric Oxide	NO^{\cdot}
Non radical oxidants	
Hydrogen peroxide	H_2O_2
Organic peroxide	$ROOH$
Hypochlorous acid	$HOCL$
Ozone	O_3
Aldehydes	$HCOR$
Singlet oxygen	1O_2
Peroxynitrite	$ONOOH$

Table 1. Radical and non-radical oxidants.

ROS are frequently converted into each other, for example, superoxide into peroxide (by dismutase enzymes) and peroxide into hydroxyl radicals (a metal-catalysed Fenton reaction). Reactions of ROS with themselves or other molecules also result in secondary reactive species, such as peroxynitrite, the condensation product of superoxide and nitric oxide radicals and a range of reactive sulfur species formed from ROS and cysteine residues. In addition, ROS attack on metalloproteins frequently results in the release of their metal ions, such as Zn^{2+} , and redox-active $Fe^{2+/3+}$ and $Cu^{+/2+}$. The latter two are able to participate in Fenton-type reactions (a), that is, Fe^{2+} and Cu^+ reduce H_2O_2 to form HO^{\cdot} (Winyard et al., 2005).



Most of the transition metals contain unpaired electrons and can, therefore, with the exception of zinc, be considered radicals by definition. They can participate in the chemistry of radicals and convert relatively stable oxidants into powerful radicals. Among the various transition metals, copper and especially iron are present in relatively high concentrations and are major players in the Fenton reaction and the metal-mediated Haber-Weiss reaction. The metal ions participating in this reaction are those bound to the surface of proteins, DNA, and other macromolecules or chelates. These particular ions can still undergo the

reduction-oxidation process, interacting with oxygen derivatives (Kohen and Nyska, 2002).

All cells living under aerobic conditions are continuously exposed to large numbers of oxidants derived from various endogenous and exogenous sources (Halliwell and Gutteridge, 1999). The endogenous sources of oxidants are several and include the respiratory chain in the mitochondria that converts molecular oxygen to water. A few percent of oxygen molecules continuously leaks from the electron transport chain as ROS intermediates and this process alone results in a substantial basal level of oxidants *in vivo*. In addition, immune reactions may contribute considerably to the generation of oxidants, in particular during infections or as a result of autoimmune responses. For example, activated neutrophils undergoing the respiratory burst release ROS intended to target foreign pathogens, but the lack of specificity in these reactions also results in tissue damage to the host. Furthermore, enzymes such as xanthine oxidase and nitric oxide synthase produce $O_2^{\cdot-}$ and NO^{\cdot} , two radicals that can energetically combine to form the deleterious species $ONOO^{\cdot}$ (Halliwell, 2009). Moreover, foreign microorganisms induce secondary oxidant formation and release in the host via the immune system, as mentioned above, in addition to their sometimes directly oxidising capabilities (Lykkesfeldt and Svendsen, 2007).

1.1.2 Oxidative damage

Oxidative stress, as previously reported, is commonly defined as an imbalance between oxidants and reductants (antioxidants) at the cellular or individual level. Oxidative damage is one result of such an imbalance and includes oxidative modification of cellular macromolecules, cell death by apoptosis or necrosis, as well as structural tissue damage. The presence of free radicals and non-radical reactive molecules at high concentrations is dangerous because of their ability to damage cell organelles. Nitric monoxide (NO), superoxide anions, ROS and nitrogen species (RNS), however, also play important modulating roles in certain signal transduction pathways. Several ROS-mediated reactions protect the cell from oxidative stress and serve to stabilize redox homeostasis. In more developed organisms NO and ROS act as signal transducing molecules, modulating vascular tone, monitoring oxygen pressure and production of erythropoietin, as well as playing a role in signal transduction pathways involving membrane receptors as part of various physiological processes (Somogyi et al., 2007).

Because most radicals are short-lived species, they react quickly with other molecules. Some of the oxygen-derived radicals are extremely reactive with a short half-life. The life span of other radicals is also short but depends on the environmental medium. Non-radical metabolites also possess a relatively short

half-life varying from parts of seconds to hours, as in the case of HClO. The physiological environment, consisting of such factors as pH and the presence of other species, has a great influence on the half-life of ROS. The high reactivity of radicals and their short life span illustrate the potential toxic effect and difficulties in preventing oxidative damage. To prevent the interaction between radicals and biological targets, the antioxidant should be present at the location where the radicals are being produced in order to compete with the radical for the biological substrate (Kohen and Nyska, 2002).

The cellular macromolecules, in particular DNA, proteins and lipids, are natural targets of oxidation.

Although DNA is a stable, well-protected molecule, ROS can interact with it and cause several types of damage: modification of DNA bases, single- and double-DNA breaks, loss of purines (apurinic sites), damage to the deoxyribose sugar, DNA-protein cross-linkage, and damage to the DNA repair system. Not all ROS can cause damage; most is attributable to hydroxyl radicals (Kohen and Nyska, 2002). It is estimated that ROS are responsible for about 10 000 DNA base modifications per cell per day. Oxidation or methylation of bases is thought to have the most serious phenotypic consequences. Mitochondrial DNA appears to be particularly vulnerable, in part due to its proximity to the site of most uncontrolled ROS generation, and because of the low level of repair that occurs. Telomeres, the caps at the chromosome end that are critical for genome stability, are also vulnerable to attack from ROS, and the accelerated reduction in telomere length that results from oxidative stress can hasten cell senescence (Monaghan et al., 2009).

Proteins can undergo direct and indirect damage following interaction with ROS, including peroxidation, damage to specific amino acid residues, changes in their tertiary structure, degradation, and fragmentation. The magnitude of the damage will depend in part on the location of the proteins relative to the site of ROS generation, and their composition and structure (Dröge, 2002). Some amino acids, notably tryptophan, tyrosine, histidine and cysteine, are much more susceptible to oxidation than others, and ROS can also alter the secondary and tertiary structure of proteins (Dröge, 2002).

Among the various ROS, the OH[•], RO[•], and RNS predominantly cause protein damage. The consequences of protein damage as a response mechanism to stress are loss of enzymatic activity, altered cellular functions such as energy production, interference with the creation of membrane potentials and changes in the type and level of cellular proteins (Kohen and Nyska, 2002). Following protein oxidation, modified proteins are susceptible to many changes in their

function. These include chemical fragmentation, inactivation, and increased proteolytic degradation (Grune, 1997).

Damage to lipids is also of great significance, as this can have major consequences for membrane structure and function in particular. Membrane composition, which is very important to the membrane function and possibly to metabolic rate, influences susceptibility to oxidative damage. Polyunsaturated fatty acids (PUFA) are much less resistant to peroxidation than monounsaturated or saturated fatty acids, and so variation in the proportion of PUFA in membranes can influence the rate of oxidative damage. PUFA are easily oxidized and may initiate chain reactions resulting in further oxidative damage, which can compromise the integrity of the cell. In this process, abstraction of a hydrogen atom by a ROS results in conjugated diene formation, which renders the lipid more susceptible to further oxidation. Its subsequent reaction with molecular oxygen results in formation of a lipid peroxy radical capable of oxidising a neighbouring lipid and thus propagating the oxidative damage, involving a range of reactive intermediates that can then also cause protein and DNA damage (Lykkesfeldt and Svendsen, 2007).

1.1.3 Involvement of oxidative stress in farm animal diseases

Oxidative stress is the cause or consequence of hundreds of diseases, in both acute and chronic conditions, from diabetes to Alzheimer's disease. A large amount of studies documented the role of oxidative stress in human diseases, few studies about oxidative stress in farm animals are available in literature. Oxidative stress in farm animal diseases has primarily been studied in pigs, cattle and horses, with a focus on ascorbate levels and NO[•] production. Studies in pigs and cattle have been somewhat sporadic and mainly with infectious diseases, such as pneumonia, enteritis, mastitis, endometritis and sepsis. Studies in horses, in particular racing horses and horses with airway obstruction have been more systematic, including recurrent airway obstruction, exercise-induced pulmonary haemorrhage, racing-induced oxidative stress, laminitis, arthritis and intestinal strangulation (Lykkesfeldt and Svendsen, 2007).

Pigs diseases

Pneumonia. Piglets and adult pigs have been used as models of the potential toxicity of combined treatment of human neonates with inhalation of high levels of O₂ and NO[•] (Robbins et al., 1995) and for evaluation of a potential anti-inflammatory effect of inhaled NO[•] in a porcine model of cardiopulmonary bypass-induced pulmonary inflammation (El Kebir et al., 2005).

Enteritis. Physiologically, enteric nervous system release of NO[•] plays a role in propagation of intestinal contents, and vascular release of NO[•] influences

intestinal blood flow. The activity of a biomarker of granulocyte infiltration and intestinal inflammation, myeloperoxidase, paralleled the increase in nitrite levels. The nitric oxide synthase (NOS) inhibitor L-NAME reduced epithelial permeability. This suggests that intestinal nitrite production may be a useful biomarker of gut injury (Lykkesfeldt and Svendsen, 2007).

Sepsis. The sepsis syndrome in piglets is a relatively frequent event, characterised by altered vascular tone and organ perfusion and, in particular, pathologically elevated pulmonary pressure. Intestinal motility disturbances are also associated with sepsis. Porcine endotoxaemic shock seems to be associated with increased oxidative stress and damage. Basu et al. (2000) found decreased antioxidant status, as measured by α -tocopherol, and increased lipid peroxidation, as measured by isoprostanes, in pigs with endotoxaemia. Eight isoprostanes were measured as a biomarker of lipid peroxidation and NOS inhibition attenuated the sepsis-induced increase in oxidative damage, thereby supporting a role for NO \cdot in the oxidative stress observed in sepsis (Matejovic et al., 2004).

1.2 The antioxidant defence

The cellular defence mechanisms against oxidants can be divided into at least three levels according to their function of quenching oxidants, repairing oxidative damage or encapsulating non-repairable damage. As a first level of defence against oxidants, the cell is equipped with a so-called antioxidant network. Antioxidants are capable of donating electrons to oxidants, thus quenching their reactivity under controlled conditions and making them harmless to cellular macromolecules. The antioxidants thereby become radicals themselves, but these are far more stable and are not capable of inducing cellular damage. The oxidised antioxidants are subsequently recycled to their active reduced state by a number of efficient cellular processes fuelled by energy from NADPH. This recycling is the key to the power of the antioxidant network, which would otherwise deteriorate rapidly (Lykkesfeldt et al., 2003). The antioxidant network can be classified into two primary groups, the low molecular weight and high molecular weight (enzymatic) antioxidants (Lykkesfeldt and Svendsen, 2007).

Antioxidants have different localization depending on their chemical characteristics. Intracellular antioxidants include low molecular weight scavengers of oxidizing species and enzymes which degrade superoxide and hydroperoxides. Their chemical characters define their localization in the cells. Hydrophilic chain breakers are found in cytosolic, mitochondrial and nuclear compartments. Hydrophobic chain breaking antioxidants are found in cell membranes where they inhibit or interrupt chain reactions of lipid peroxidation. Enzymatic compounds are primarily responsible for intracellular defence. Non-

enzymatic compounds, such as uric acid and bilirubin, as well as a certain group of vitamins (based on their significant chain breaking capabilities) are among the most important non-enzymatic antioxidant compounds that can be found in blood plasma (Blokhina et al., 2003; Fang et al., 2002). Non-vitamin antioxidant compounds (uric acid, bilirubin, albumin etc) are responsible for 0–80% of the cumulative chain breaking capability possessed by blood plasma. Other antioxidant compounds (e.g. coeruloplasmine, transferrin, thiols) only play a limited role in antioxidant defence, since their concentration in blood plasma is low. (Somogyi et al., 2007)

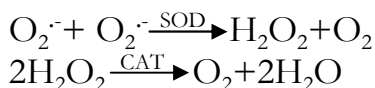
Some of these antioxidants are endogenously produced which include enzymes, low molecular weight molecules and enzyme cofactors, others are introduced with the diet.

1.2.1 High molecular weight antioxidants

High molecular weight antioxidants (enzymatic antioxidants) include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), etc. Antioxidant enzymes, SOD and CAT, are not consumed and have high affinity and rate of reaction with ROS. SOD and CAT are among the most potent antioxidants known in nature.

The proteins in the SOD-family differ in their structure and cofactors. Cu-Zn SOD is an enzyme of molecular mass of approximately 32 kD; it contains two subunits, each of which possesses an active site; it is widely distributed in eukaryotic cells localized in the cytoplasm, while Mn-SOD, a protein of about 40 kD, can be found in prokaryotic cells and eukaryotic mitochondria. Other types of SOD exist, such as extracellular SOD (EC-SOD) and Fe-SOD in plants. These enzymes possess different structures, molecular masses, and reaction rate constants. The enzyme activity itself is capable of enhancing the spontaneous dismutation of superoxide radicals to H₂O₂. The end product of the dismutation reaction can be removed by the activity of CAT and members of the peroxidase family including GPx (Kohen and Nyska, 2002).

CAT can remove H₂O₂ present in high concentrations. The enzyme consists of 4 protein subunits, each of which contains ferric ions of the heme group that undergo oxidation following interaction with H₂O₂.



In contrast to catalase, peroxidase possesses high affinity for and can remove H₂O₂ even when it is present in low concentration. The removal of H₂O₂ is an expensive reaction as it consumes valuable molecules in the cellular

environment; two molecules of glutathione are consumed for the removal of one molecule of H₂O₂.



Other enzymes exist in the cellular environment that support the activity of antioxidant enzymes. For example glucose-6-phosphate dehydrogenase supplies reducing equivalents (NADPH) necessary for cellular function and important for the regeneration of oxidized antioxidants; the regeneration of oxidized glutathione, GSSG, to the reduced form, GSH, by reduced nicotinamide dinucleotide (NADH) is but one example. Another supporting enzyme is xanthine dehydrogenase, that produces uric acid, an effective endogenous antioxidant (Kohen and Nyska, 2002).

1.2.2 Low molecular weight antioxidants

The low-molecular-weight antioxidant (LMWA) group contains numerous compounds capable of preventing oxidative damage by direct and indirect interaction with ROS. The indirect mechanism involves the chelation of transition metals that prevents them from participating in the metal-mediated Haber-Weiss reaction (Samuni et al., 1983). The direct acting molecules share a similar chemical trait that allows them to donate electrons to the oxygen radical so that they can scavenge the radical and prevent it from attacking the biological target. Scavengers possess many advantages over the group of enzymatic antioxidants. Because scavengers are small molecules, they can penetrate cellular membranes and be localized in close proximity to the biological target. The cell can regulate their concentrations, and they can be regenerated within the cell. They possess a wide spectrum of activities toward a large variety of ROS. The scavenging mechanism can proceed only if the concentration of the scavenger is sufficiently high to compete with the biological target on the deleterious species (Kohen and Gati, 2000). Scavengers originate from endogenous sources, such as biosynthetic processes and waste-product generation by the cell, and exogenously from diet. The number of LMWA synthesized by the living cell or generated as waste products is limited (eg, glutathione, uric acid, lipoic acid, and bilirubin); most LMWA are derived from dietary sources. Scavengers are characterized by their common mechanism of activity, reacting directly with the radical and removing it by donating an electron(s) to the reactive species. This reaction results in the conversion of the scavenger by itself to a radical, although not a reactive one. The scavenger radical can undergo further oxidation or be regenerated to its reduced form, a reducing antioxidant, by another scavenger possessing a suitable oxidation potential; the ascorbyl radical, for instance, can be recycled to its reduced form, ascorbic acid, by glutathione. The regeneration

process can be purely chemical, or an enzyme can be involved in the electron transfer.

The principal LMWA antioxidants are:

- Glutathione: it is a low-molecular-mass, thiol-containing tripeptide, glutamic acid-cysteine-glycine (GSH) in its reduced form and GSSG in its oxidized form, in which 2 GSH molecules join via the oxidation of the SH groups of the cysteine residue to form a disulphide bridge. It acts as a cofactor for the enzyme peroxidase, thus serving as an indirect antioxidant donating the electrons necessary for its decomposition of H_2O_2 . In addition it can scavenge ROS directly interacting with $OH\cdot$, $ROO\cdot$, and $RO\cdot$ radicals; upon reaction with ROS, it becomes a glutathione radical, which can be regenerated to its reduced form;
- Uric acid: it is a cellular waste product originating from the oxidation of hypoxanthine and xanthine by xanthine oxidase and dehydrogenase. Urate, the physiological state of uric acid, reacts with hydroxyl radicals producing a stable urate radical that can be regenerated by ascorbate to its prior state, urate. This compound can act with peroxy radicals, 1O_2 , O_3 , $NO\cdot$, and other RNS. Urate also protects protein from nitration; it can chelate metal ions, such as copper and iron, and prevent them from participating in redox cycling;
- Ascorbic acid: it is a water-soluble antioxidant that can be synthesized by plants and some animals. Humans, primates, guinea pigs, and fruit bats have lost the enzyme needed for its synthesis. As an antioxidant, ascorbate is an efficient scavenger, or reducing antioxidant, capable of donating its electrons to ROS and eliminating them. Ascorbate can donate two electrons; following donation of one electron, it produces the ascorbyl (semidehydroascorbate or ascorbate) radical, which can be further oxidized to produce dehydroascorbate. In vitro, ascorbate can act as an efficient antioxidant and scavenge a variety of ROS including hydroxyl, peroxy and oxosulphuric radicals. Ascorbate is also a powerful scavenger of $HClO$ and peroxyxynitrous acid and can inhibit the peroxidation process.
- tocopherol antioxidants: These compounds, chain-breaking antioxidants, can scavenge $ROO\cdot$ to inhibit the lipid peroxidation process in biological membranes. Eight naturally occurring substances are known to be members of the vitamin E family. These compounds have three asymmetric carbon atoms, giving 8 optical isomers. The most effective form in animals is d- α -tocopherol. Following interaction, tocopherol is converted to tocopherolquinone and subsequently to tocopherylquinone. As with other scavengers, the α -tocopheryl radical can be recycled to its active form.

A second and highly important level of defence is the ability to detect and repair or remove oxidised and damaged molecules. Included in this part of the defence

is a series of DNA repairing enzymes capable of detecting oxidised bases or misincorporations, cutting them out and inserting the correct undamaged base in the DNA. Other means of second level defence include catabolism of nonfunctional or modified proteins and lipids. Finally, if the extent of the oxidative damage exceeds the capacity of repair and removal, cells undergo apoptosis. The ability to induce programmed cell death is of major importance in a variety of functions, including control of tissue growth, and is apparently under control by several signalling pathways. However, one of these appears to be that apoptosis is induced by increased oxidative stress and thus constitutes a final resort to encapsulate and isolate the damaged cells (Lykkesfeldt and Svendsen 2007).

1.2.3 Antioxidants and diet

The dietary intake of antioxidants is thought to play a major role in the antioxidant network. The indirect antioxidant effect (upregulation of the antioxidant defense or repair systems) may be evoked by xenobiotics or components in vegetables that are not scavengers or even considered harmful; e.g., isothiocyanates are oxidants that stimulate cellular antioxidant proteins and detoxification enzymes. Antioxidants such as vitamin C, vitamin E, carotenoids, and flavonoids have been identified in many natural food products. Natural products also contain mixtures of other antioxidants and bioactive substances with unknown antioxidant properties (Moller and Loft, 2006). Plants contain high concentrations of numerous redox-active antioxidants, such as polyphenols, carotenoids, tocopherols, glutathione, ascorbic acid and enzymes with antioxidant activity, which fight against hazardous oxidative damage of plant cell components. In animal cells, antioxidant production is much more limited. Therefore, plant-sourced food antioxidants like vitamin C, vitamin E, carotenes, phenolic acids, phytates and phytoestrogenes have been recognized as having the potential to reduce oxidative damage in animals. The intake of food rich in α -tocopherols, β -carotene and ascorbic acid has been associated with reduced oxidative-stress related diseases. Phenolic acids, polyphenols and flavonoids scavenge free radicals such as peroxide, hydroperoxide or lipid peroxy, thus inhibiting the oxidative mechanism that lead to degenerative diseases (Pisoschi et al., 2009).

Many studies have reported that dietary supplements such as antioxidants, vitamins, and minerals prevent or at least attenuate organic impairment originated by excess oxidative stress (Girard et al., 2005). Inhibition of oxidative damage by supplementation of antioxidants becomes an attractive therapeutic strategy to reduce the risk of these diseases (Dai et al., 2006). There are, in addition, several nutritionally essential minerals incorporated into protective antioxidant enzymes. Zinc, copper, and manganese are required for activity of

the two types of superoxide dismutases. Selenium, an essential component of glutathione peroxidase, is important in the decomposition of hydrogen peroxide and lipid peroxides. The level of dietary intake of all the antioxidant micronutrients directly affects the circulating level of these nutrients and the activity of the antioxidant metalloenzymes. Thus, low intakes of one or more of these antioxidant nutrients could reduce the body's defenses against free radical damage and increase susceptibility health problems associated with free radical damage (Machlin and Bendich, 1987).

The use of antibiotics as growth promoters in animal feeds is facing reduced social acceptance due to the appearance of residues and resistant strains of bacteria. The use of antibiotics has been prohibited in the European Union since January 2006 (Regulation 1831/2003/EC). Natural, safe and inexpensive feed additives that do not endanger the environment with residues in wastes should be developed. There has been a revival of phytogetic feed additives (PFA) as a successful alternative to the prophylactic use of antibiotics. The action of PFA is a result of natural substances that contain low-molecular-weight reactive oxygen species-scavenging substances, where polyphenols are included among these. PFA influenced positively daily feed intake, daily weight gain, and feed utilization in growing pigs and improved growth performance in pigs (Wang et al., 2008). Most of these active secondary plant metabolites belong to the classes of isoprene derivatives, flavonoides and glucosinolates, and a large number of these compounds has been suggested to act as antibiotics or as antioxidants *in vivo* as well as in food (Wenk et al., 2003). Some antioxidants are used to protect the nutrients in the feed during storage. Others have their main activity in the digestive tract where they may also help that the substances sensible for oxidation can be absorbed. In the intermediate metabolism antioxidants are responsible for many functions like reduced aging or the protection of intact membranes. In farm animals antioxidants can have a direct influence on the product quality (Wenk et al., 2003). Amrik e Bilkey (2004) and Khajarerne Khajarerne (2002) found an improvement in productive performance, feed conversion and feed intake in sow fed with oregano; Kyriakis et al., (1998) found that medication with oil of *Origanum* was effective in controlling postweaning diarrhoea syndrome in piglets and also reduced the mortality rate. Use of antioxidant in animal feeding reduces lipid oxidation in meat, responsible of color, flavor, nutritive value and in general improves meat pork (Jensen et al., 1998; Mohanan et al., 1990; Mason et al., 2005; Corino et al., 1999; 2007), chicken (Botsoglou et al., 2002) and rabbit (Bostoglou et al., 2004) quality.

Flavonoids

Flavonoids are polyphenolic compounds which are widespread in foods and beverages and possess a wide range of biological activities, of which antioxidation has been extensively demonstrated (Dai et al., 2006). Flavonoids, have a polyphenol structure, which contains numerous double bonds and hydroxyl groups that can donate electrons through resonance to stabilize the free radicals (Machlin and Bendich 1987). There are several subclasses of flavonoids: flavanols, flavanones, flavones, isoflavones, anthocyanidins, and flavonols. The divisions in flavonoid subclasses are based on structural properties. The plants, and thus foods they are found in differ, as well. The flavanols are found in red grapes and red wine, flavanones are in citrus foods, flavones are in green leafy spices, isoflavones are found in soy foods, anthocyanidins are in berries, and flavonols are found in almost all foods (Bentz, 2009). The structures of flavonoids differ greatly within the major classifications and substitutions include glycosylation, hydrogenation, hydroxylation, malonylation, methylation, and sulfation. The pattern of conjugation, glycosylation, or methylation can be very complex, can modify the hydrophilicity of the molecule and its biological properties. Flavonoid molecules not attached to sugar moieties are referred to as the aglycone form, whereas flavonoid molecules with sugar moieties are called flavonoid glycosides. Except for catechins, flavonoids do not occur in plants as aglycones; the most frequently occurring forms are the glycoside derivatives in plants. Glycosylation increases the polarity of the flavonoid molecule, which is necessary for storage in plant cell vacuoles. Flavonols and flavones occur in food usually as α - β -glycosides (Aherne and O'Brien, 2002).

Phenylpropanoid glycosides are widely distributed in different plant families. Various plants used in traditional medicine contain significant amounts of these compounds. Some of these phenylpropanoid glycosides are considered to have biological properties such as antibacterial, antiviral, analgesic, antispasmodic, neuroprotective, cytostatic, anti-inflammatory and strong radical scavenger activity (Diaz et al., 2004).

Among polyphenols, verbascoside (Fig.1), a phenylpropanoids glycoside (PPG) shows many biological activity of interest. It shows the highest scavenger activity inside the PPG tested (Wang et al., 1996) and it has a high antioxidant power also in comparison with other classes of phenolic compounds. More recently Rossi et al. (2009) found that verbascoside has greater antioxidant power compared to other phenolic compounds and Trolox.

It has two catecolic functions and its aglycone (caffeic acid) has a strong antioxidant activity.

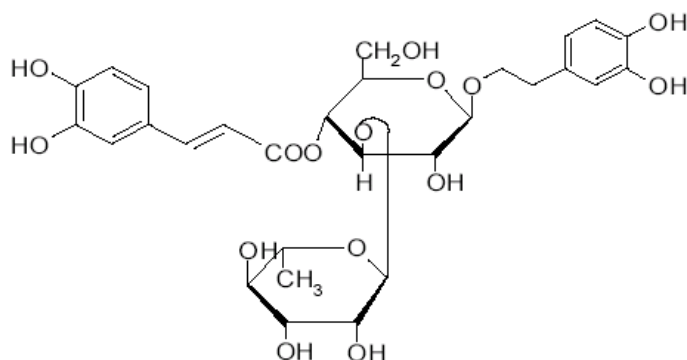


Fig.1. Verbascoside chemical structure.

Verbascoside occurs in many plants belonging to the Bignoniaceae, Buddlejaceae, Gesneriaceae, Labiatae, Oleaceae, Orobanchaceae, Scrophulariaceae, and Yerbenaceae; it is produced by plant cell cultures, particularly by *Syringa vulgaris* (Oleaceae) cell cultures. In a study on natural polyphenols and on other compounds verbascoside showed the highest activity versus the oxidation of protein induced by peroxide radical (Salvi et al., 2002). Other properties of interest are anticancer activity: inhibition of protein kinase C, topoisomerase II and telomerase; induction of apoptosis in HL-60 (Deepak et al., 1999; Zang et al., 2002; Li et al., 1997); anti-inflammatory activity: selective inhibition of 5-lipoxygenase and protein kinase C, enzymes involved in anti-inflammatory process (Diaz et al., 2004); antibacterial activity (Ellis, 1983); cardioprotective activity (Pennacchio et al., 1996); immunomodulation activity (Akbar et al., 2002); hepatoprotective activity (Lee et al., 2004); the reduction of oxidative stress induced by Matrix metalloproteinases (MMP) in neuronal cell PC12 (Sheng et al., 2002).

1.3 Methods to assess oxidative stress

It is possible to measure oxidative stress directly, detecting free radical production, or indirectly detecting antioxidant defences or molecules marker of oxidative damage. The battery of methods available to assess any of these components of oxidative stress is enormous (Monaghan et al., 2009).

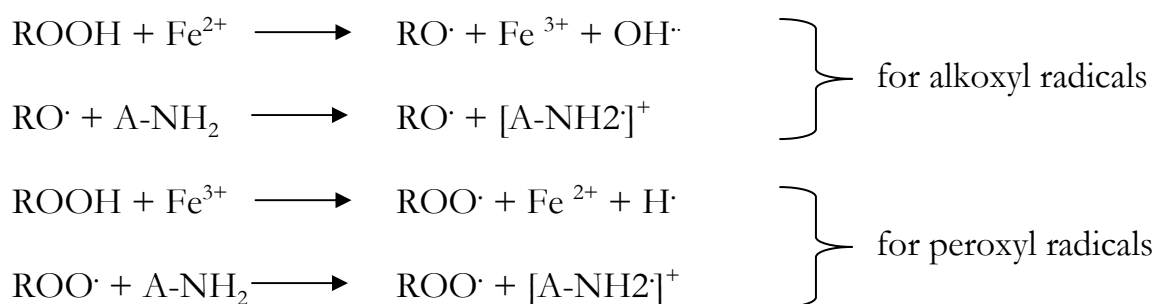
1.3.1 Free radical production

The direct analysis of ROS in biological material is difficult because of their intrinsic reactivity and short half-lives. The only technique that allows direct observation of free radicals is electron spin resonance (ESR) (Halliwell and Gutteridge, 2007). However, this technique is only able to detect the less-reactive radical species. To overcome this limitation, ESR is commonly used in combination with the technique of spin trapping, in which a trap molecule is allowed to react with a radical to produce a more stable and measurable product.

An easier approach is the detection of Reactive Oxygen Metabolites (ROMs). Because their greatest reactivity, free radicals have the potential to react with any organic molecule, thus generating a class of compounds which are more stable compared to free radicals and, therefore, they can be adequately detected and quantified.

ROMs test

In the ROMs test, ROMs (mainly hydroperoxides, ROOH) of a blood sample, in presence of iron (that is released from plasma proteins by an acidic buffer), are able to generate alkoxy ($\text{RO}\cdot$) and peroxy ($\text{ROO}\cdot$) radicals, according to the Fenton's reaction. Such radicals, in turn, are able to oxidize an alkylsubstituted aromatic amine (A-NH_2 , that is dissolved in a chromogenic mixture) thus transforming them in a pink-coloured derivative ($[\text{A-NH}_2]^+$), according to the reactions:



Finally, this coloured-derivative is photometrically quantified. Indeed, the intensity of developed colour is directly proportional to the concentration of ROMs (Iorio, 2004).

1.3.2 *Antioxidant defences*

The concentration of individual enzymatic and non-enzymatic antioxidants can be measured in different tissues and fluids. However, the antioxidant capacity is not a simple additive function of the concentration of individual antioxidants in a sample because of the synergistic and antagonistic interactions among antioxidants. Data on measurements of individual antioxidant concentrations are generally more difficult to interpret than estimations of overall antioxidant capacity of a sample against a radical attack (Cohen et al., 2007; Somogyi et al., 2007). Thus, the total antioxidant capacity (TAC) may give more biologically relevant information than that obtained from measuring concentrations of individual antioxidants. In addition, the antioxidant capacity of the cell is mainly attributable to the enzyme system, whereas that of plasma is mostly accounted for by low molecular weight antioxidants of dietary origin. These compounds, rapidly consumed during the scavenging of ROS, need to be regenerated or replaced by new dietary-derived compounds. Thus, plasma antioxidant capacity is modulated either by radical overload or by the intake of dietary antioxidants and can therefore be regarded as more representative of the *in vivo* balance between oxidizing species and antioxidant compounds than the concentration of single, selected antioxidants (Ghiselli et al., 2000).

The antioxidant capacity is the measure of the moles of a given free radical scavenged by a test solution, independently from the antioxidant activity of any one antioxidant present in the mixture. In biological samples such as plasma, a number of heterogeneous compounds displaying diverse antioxidant activity are present. Measuring plasma antioxidant capacity may help to evaluate physiological, environmental and nutritional factors that influence the redox status in humans (Somogyi, 2007).

Several methods for the measurement of TAC are available: the chemical principles of antioxidant capacity assays depend upon the reactions involved. The assays can be classified into two types: assays based on hydrogen atom transfer (HAT) reactions and assays based on electron transfer (ET).

The majority of the **HAT-based assays** apply a competitive reaction scheme, in which antioxidant and substrate compete for thermally generated peroxy radicals through the decomposition of azo-compounds.

In general, these assays apply a thermal radical generator to give a steady flux of peroxy radicals in air-saturated solution. Added antioxidant competes with probes for the radicals and inhibits or retards the probe oxidation. Assays with

this feature include total radical trapping antioxidant parameter (TRAP) assay, oxygen radical absorbance capacity (ORAC) assay, and crocin bleaching assay. These assays have the following components: (a) an azo radical initiator; (b) a molecular probe (UV or fluorescence) for monitoring reaction progress; (c) antioxidant; and (d) reaction kinetic parameters collected for antioxidant capacity quantitation (Huang et al., 2005).

ORAC assay

The ORAC assay (oxygen radical absorption capacity) was originally developed by Cao and Prior (1998). The assay measures the effectiveness of various natural antioxidants, present in plasma or tissue homogenates, in preventing the peroxy-radical-induced oxidation of the fluorescent marker protein, fluorescein. This method is based on the inhibition of peroxyradical-induced oxidation by antioxidants, which can be detected as loss in the fluorescence intensity, during peroxy-radical-induced free radical damage; thus this method is not able to measure a only a single antioxidant. Thermo decomposition of azo-compounds, such as 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH), is used as a peroxy radical generator (Fig.2). (See also KRL Test, paragraph 1.4)

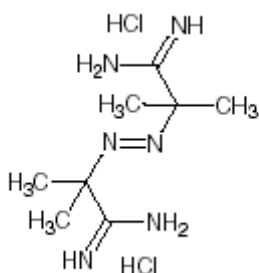


Fig.2 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH).

This method was limited to hydrophilic antioxidants due to the aqueous environment. Methylated beta-cyclodextrin (RMCD) can be used as water solubility enhancer for lipophilic antioxidants.

TRAP assay

The original total radical trapping antioxidant parameter (TRAP) method was developed by Wayner et al, (1985). Their test is based on the measure of oxygen consumption during a controlled lipid peroxidation reaction induced by thermal decomposition of an azo-compound. It was the most widely used method for measuring total antioxidant capacity of plasma or serum. The TRAP assay uses peroxy radicals generated from AAPH as initiator of free radical generation. After adding AAPH to the plasma, the oxidation of the oxidizable materials is monitored by measuring the oxygen consumed during the reaction. During an

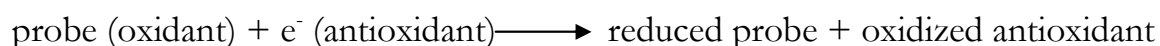
induction period, this oxidation is inhibited by the antioxidants in the plasma. An earlier detection method used the principle that peroxy radicals produced from AAPH oxidize luminol, which led to the formation of luminol radicals that emitted light. The emitted light used to be detected by a luminometer. The light emission is sensitive to interference by antioxidants. The length of the induction period (lag phase) is compared to that of an internal standard, Trolox C (6-hydroxyl-2,5,7,8,-tetramethylchroman-2-carboxylic acid), a water-soluble vitamin E analogue, and then quantitatively related to the antioxidant capacity of the plasma. The antioxidant capacity of an unknown sample was expressed as Trolox equivalence (X) by the equation:

$$C_{\text{Trolox}} / T_{\text{Trolox}} = X / T_{\text{plasma}}$$

where C_{Trolox} is Trolox concentration, T_{Trolox} is the lag time of the kinetic curve of the fluorescent probe in the presence of Trolox, X is the antioxidant capacity of plasma, and T_{plasma} is the lag time the kinetic curve in the presence of plasma. X is then multiplied by 2.0 (the stoichiometric factor of Trolox) and by the dilution factor of the sample to give the TRAP value ($\mu\text{mol/L}$). To obtain the T_{Trolox} from the same kinetic curve of the sample, Trolox was added to the reaction mixture when the probe fluorescence was $\cong 50\%$ of the initial value. The reaction was followed until the fluorescence decay rate resumed to the level before the Trolox addition. The lag phase was then calculated by extrapolating the curves of maximal fluorescent probe oxidation before and after Trolox addition (Huang et al., 2005).

The **ET-based assays** measure the capacity of an antioxidant in the reduction of an oxidant, which changes colour when reduced.

These methods involve two components in the reaction mixture, antioxidants and oxidant (also the probe). They are based on the following electrontransfer reaction:



The probe itself is an oxidant that abstracts an electron from the antioxidant, causing color changes of the probe. The degree of the color change is proportional to the antioxidant concentrations. The reaction end point is reached when color change stops. The change of absorbance is plotted against the antioxidant concentration to give a linear curve. The slope of the curve reflects the antioxidant's reducing capacity, which is expressed as Trolox equivalence (TE) or gallic acid equivalent (GAE) (Huang et al., 2005).

FRAP assay

The FRAP assay (ferric reducing antioxidant power) (Benzie and Strain, 1996; 1999, Ozgen et al., 2006) measures antioxidant power with the help of an oxidant, i.e., Fe^{3+} . Ferric to ferrous ion reduction at low pH causes the formation of a coloured ferrous–tripyridyltriazine complex. FRAP values are obtained by comparing the absorbance change at 593 nm in test reaction mixtures with those containing ferrous ions in known concentration. In the FRAP assay, antioxidants in the sample reduce the Fe(III) /tripyridyltriazine complex, present in stoichiometric excess, to the blue ferrous form, with an increase in absorbance at 593 nm. The change in absorbance is proportional to the combined (total) ferric reducing/antioxidant power (FRAP value) of the antioxidants in the sample (Ou et al., 2002). Absorbance changes are linear over a wide concentration range with antioxidant mixtures, including plasma, and with solutions containing one antioxidant in purified form. Since there are no free radicals introduced into the system, there is no way of comparing the antioxidant capacity towards different kinds of radicals.

TEAC assay

The Trolox equivalent antioxidant capacity (TEAC) assay was first reported by Miller et al (1993), and then modified by Re et al. (1999). When 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) is incubated in the presence of a peroxidase and hydrogen peroxide or in the presence of hydroxyl, peroxy, alkoxy and inorganic radicals, ABTS+ radical cation is generated (Fig.3). As the ABTS+ radical cation begins to form, the absorbance increases.

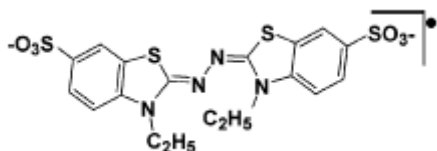


Fig.3 ABTS+ radical cation

When antioxidants are added before the addition of hydrogen peroxide, the antioxidants scavenge the radicals formed by the hydrogen peroxide, delaying the formation of the ABTS+ radical cation, thus inducing an increase in the percentage of inhibition of the absorbance. The TEAC assay is based on the inhibition by antioxidants of the absorbance of the radical cation of ABTS, which has a characteristic long wavelength absorption spectrum showing maxima at 660, 734 and 820 nm.

TOSC assay

The total oxidant scavenging capacity (TOSC) assay (Winston et al 1998) permits quantification of the absorbance capacity of antioxidants towards three potent oxidants, i.e. hydroxyl radicals, peroxy radicals and peroxyxynitrite. These oxidants were generated by the iron plus ascorbate-driven Fenton reaction, thermal homolysis of AAPH and 3-morpholinolinosydnonimine N-ethylcarbamide (SIN-1), respectively. These oxidants react with alpha-keto gamma-methiolbutyric acid (KMBA), which is oxidized and yields ethylene. The antioxidant capacity of the compounds tested is quantified from their ability to inhibit ethylene formation relative to a control reaction. Thus, the relative efficiency of various antioxidants could be compared under conditions of quantitatively similar KMBA oxidizing capability by the three oxidants. The TOSC assay is useful and robust in distinguishing between the reactivities of various oxidants and the relative capacities of antioxidants to scavenge these oxidants (Regoli and Winston, 1999; Winston et al., 1998).

DPPH assay

2,2-Diphenyl-1-picrylhydrazyl radical (DPPH) is one of a few stable and commercially available organic nitrogen radicals and has a UV-vis absorption maximum at 515 nm (Fig.4). Upon reduction, the solution color fades; the reaction progress is conveniently monitored by a spectrophotometer.

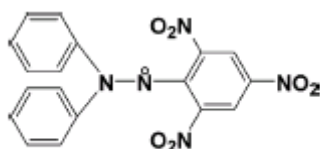


Fig.4 2,2-Diphenyl-1-picrylhydrazyl radical (DPPH).

The DPPH assay is typically run by the following procedure: DPPH solution (3.9 mL, 25 mg/L) in methanol is mixed with sample solution (0.1 mL). The reaction progress absorbance the mixture is monitored at 515 nm for 30 min or until the absorbance is stable. Upon reduction, the color of the solution fades. The percentage of the DPPH remaining is proportional to the antioxidant concentrations, and the concentration that causes a decrease in the initial DPPH concentration by 50% is defined as EC_{50} . The time needed to reach the steady state with EC_{50} concentration is calculated from the kinetic curve and defined as TEC_{50} (Somogyi et al., 2007).

CUPRAC assay

Apak et al. (2005) developed a cupric reducing antioxidant capacity (CUPRAC) spectrophotometric method for a number of polyphenols and flavonoids using

the copper(II)-neocuproine reagent in ammonium acetate buffer applied to a complete series of plasma antioxidants for the assay of TAC of serum, and the resulting absorbance at 450nm was recorded either directly (e.g. for ascorbic acid, α -tocopherol and glutathione) or after incubation at 50°C for 20 min (e.g. for uric acid, bilirubin and albumin), quantitation being made by means of a calibration curve. The lipophilic antioxidants, α -tocopherol and β -carotene, were assayed in dichloromethane. Lipophilic antioxidants of serum were extracted with n-hexane from an ethanolic solution of serum subjected to centrifugation. Hydrophilic antioxidants of serum were assayed after perchloric acid precipitation of proteins in the centrifugate (Apak et al., 2005).

1.3.3 Oxidative damage

Free radicals and other reactive species can cause oxidative damage to lipids, proteins and DNA. A global indicator of oxidative damage is not yet available, and thus the assessment of the oxidative status of an organism may require a combination of various methods to measure different oxidative damage biomarkers (Mateos and Bravo, 2007).

Lipid peroxidation gives rise to a number of secondary, highly damaging products. The two most used as biomarkers of lipid peroxidation are malondialdehyde (MDA) and F2-isoprostanes. Both are important secondary decomposition products of polyunsaturated fatty acid (Halliwell and Gutteridge, 2007). Several spectrophotometric and chromatographic methods have been developed for the measurement of MDA and isoprostanes (Del Rio et al., 2005; Mateos and Bravo, 2007).

Most assays to determine MDA have been developed on the basis of its derivatization with thiobarbituric acid (TBA). The condensation of MDA and TBA gives rise to a high absorbivity adduct which can be easily assessed with a spectrophotometer. Unfortunately, the specificity of the test based on this reaction is low, as TBA may react with several compounds other than MDA also derived from oxidation. Moreover, the treatment of biological samples to obtain the condensation product is usually carried out at high temperature (around 100 °C) and may generate further oxidation of the matrix with obvious overestimation of the results. To minimize matrix oxidation, most of these methods involve the precipitation of protein prior to the TBA reaction as a pre-treatment of plasma samples (Del Rio, 2005). One of the first and still most widely used methods to detect MDA is that developed by Yagi in 1976, carrying over the TBA reaction on a blood lipid and protein precipitate at 95°C in acidic conditions. Based on this method, results are often reported as “TBA reacting substances” (TBARS) instead of MDA. More refined versions of the TBARS

assay quantify only the genuine MDA-TBA derivative, e.g. by HPLC, or measure MDA directly without derivatisation (Lykkesfeldt and Svendsen, 2007).

F2-isoprostanes can be either determined by gas chromatography-based methods or using antibodies. Whereas the first method is complicated and time consuming, it provides more reliable results compared with immunological methods (Grune and Berger, 2007).

Due to its simplicity, protein oxidation is often used as a measure of oxidative damage. Proteins oxidation occurs as a result of either direct attack by ROS or indirectly through peroxidation of lipids that further degrade and attack proteins (Halliwell and Gutteridge, 2007). As a result of this oxidation, carbonyls are introduced into proteins either by direct oxidation of aminoacids or indirectly by attachment of a carbonyl-containing moiety (Mateos and Bravo 2007). Carbonyl formation often alters protein conformation, which enhances non-specific protein-protein interactions that compromise cell viability and impair protein turnover. Thus, oxidative damage to proteins leads to a loss of functional and structural efficiency, with increased levels of protein carbonyls, one of the most used biomarker of oxidative damage to proteins. Some of the methods for the detection and quantification of protein carbonyl groups include spectrophotometry, enzyme-linked immunosorbent assay (ELISA) and electrophoresis followed by Western blot (Halliwell and Gutteridge, 2007).

Oxidative damage to DNA can result in the modification of sugars and bases, deoxyribose damage, strand breakage and DNA-protein cross-links (Monaghan et al., 2009). The most commonly used biomarkers of DNA damage through modified bases are the concentrations of nucleosides 8-hydroxy-2'-deoxyguanosine (8-OHdG) and 8-hydroxyguanine. These products are the oxidized derivatives of guanine, the nucleotide most prone to oxidation (Mateos and Bravo, 2007). Both of these biomarkers show high specificity, can be relatively abundant in DNA and measure biologically important mutagenic lesion (Guertens et al., 2002; Halliwell and Gutteridge, 2007; Mateos and Bravo, 2007). High-performance liquid chromatography with electrochemical detection (HPLC-ECD) is the most common means of determining the levels of 8-OHdG or 8-OH-G. ROS attack upon guanine generates other products, and the ratio of these products to 8-OHdG will depend on the redox state of the cell and the presence of transition metal ions, thus the same amount of attack on DNA by reactive species could generate different levels of 8-OHdG (Halliwell and Gutteridge, 2007). In addition, because exposure to pro-oxidant agents can lead to different types of oxidative DNA damage, it is intrinsically unreliable to measure a single product as an index of oxidative DNA damage (Guertens et al., 2002; Halliwell and Gutteridge, 2007). A better approach is to measure multiple

modified bases in DNA at the same time, as when a gas chromatography-mass spectrometry assay is used. Other methods to assess oxidative DNA damage such as HPLC with mass spectrometry, antibody based immunoassays and the comet assay (single-cell electrophoresis method), are also available (Kow and Dare 2000; Guetens et al. 2002; Cadet et al. 2003; Mateos and Bravo 2007).

The comet assay is used for rapid detection and quantitation of DNA damage from single cells. It is based on the alkaline lysis of labile DNA at sites of damage. Cells are immobilized in a thin agarose matrix on slides and gently lysed. When subjected to electrophoresis, the unwound, relaxed DNA migrates out of the cells. After staining with a nucleic acid stain, cells that have accumulated DNA damage exhibit brightly fluorescent comets, with tails of DNA fragmentation or unwinding. In contrast, cells with normal, undamaged DNA appear as round dots, because their intact DNA does not migrate out of the cell. A general caveat for assays aimed at measuring modified DNA bases is that these methods do not tell us whether the damage occurs in active genes, inactive genes, telomeres or “junk” DNA (Monaghan et al., 2009). To evaluate the biological importance of oxidative DNA damage we need to know the location as well as the level of damage (Halliwell and Gutteridge, 2007). More recently, molecular techniques have been used to determine, for example, the effect of dietary antioxidants on the expression of several specific gene sequences related to oxidative stress (Selman et al. 2006). The combined use of techniques of analytical chemistry and molecular genetics is likely to be very productive (Monaghan et al., 2009).

1.4 KRL Test

The KRL test is a simple biological test that measures the global resistance of an organism against free radicals attack. It's a HAT based assay that permits to evaluate globally antioxidant defences of an organism testing blood samples.

Within the strategy to maintain redox balance against oxidant conditions (e.g., chronic inflammation, cigarette smoking, and diets poor in antioxidants and/or rich in pro-oxidants), blood has a central role because it transports and redistributes antioxidants to every part of the body. For example, plasma can scavenge long-lived ROS, such as the super-oxide anion or hydrogen peroxide, thus preventing reactions with catalytic metal ions to produce more harmful species. It can also reduce oxidized ascorbic acid back to ascorbate. Hence, plasma antioxidant status is the result of the interaction of many different compounds and systemic metabolic interactions (Ghiselli et al., 2000).

In addition to plasma antioxidant properties, red blood cells (RBCs) antioxidant defences have to be evaluated. Although erythrocytes contain an extensive antioxidant defense system, oxidative damage of membrane proteins and lipids contribute to the senescence of normal cells that result in a shorter life span for damaged cells (Shiva Shankar Reddy, 2007). Because of their susceptibility to peroxidation, RBCs have been used as a model to investigate oxidative damage in biomembranes. Exposure of RBCs to free radicals may lead to a number of membrane changes, including lipid peroxidation, reduction in deformability, changes in cell morphology, protein cross-linking and fragmentation (Zou et al., 2001).

KRL test has been developed by Laboratoires Spiral, Dijon, France (Prost, 1989, 1992). KRL test evaluates total antiradical capacity of blood measuring the time needed to hemolyze 50% of the RBCs exposed to a controlled free radical attack. This measure takes into account the complexity of the antioxidant defence system of both plasma and cells. RBCs analysis allows to quantify intracellular defences. The average life span of RBCs is 60-85 days, KRL analysis of RBCs antioxidant defences reflects the free radical aggression of the last two-three months, while KRL analysis of whole blood gives an idea of total antioxidant defences of the organism at the time of sampling.

The KRL test have several applications *in vivo*, particularly tested on human studies, or *in vitro*. In humans KRL is used to study the effectiveness of natural or pharmaceutical treatments or to discover acute processes such as trauma and ischemia or inflammatory disease (Lesgards et al., 2002); moreover it allows to discriminate welfare conditions depending on medium or high stress, or tobacco smoking.

KRL test description

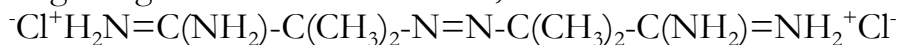
The principle of the biological test is to submit whole blood to a thermocontrolled free radical aggression in order to mobilize all families of free radical scavengers present in the blood to fight off the oxidant attack (Stocker et al., 2003; Pieri et al., 1996; Girodon et al., 1997). All chemical and enzymatic antioxidant system of the sample are activated to protect cell integrity till their lysis.

Whole Blood samples are submitted in isotonic saline solution to organic free radicals produced at 37°C under air atmosphere from the thermal decomposition of a 27 mmol/L solution of 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) (Spiral, Dijon, France).

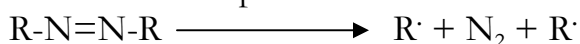
KRL test can measure the resistance to free-radical attack of only RBCs; in this case RBCs samples are obtained by centrifugation of whole blood at 1300g for 10 minutes at 4°C; after removal of plasma and buffy coat, RBCs are diluted 1:50 in isotonic buffer.

60 µl of whole blood or RBCs suspension are added to 1440 µl of dilution buffer; 50 µl of the obtained solution are dispensed in a well of a 96-well microplate containing 220 µl of AAPH solution.

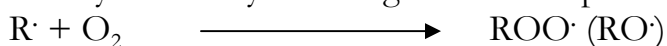
Organic generator of free radicals, AAPH:



Thermal decomposition of AAPH :



Peroxy or alkoxy radical generation in presence of oxygen:



The thermal decomposition of AAPH proceeds at a constant speed rate of 1.36 10^{-6} [AAPH] mol/l/sec, ensuring a constant free-radical flow during all the hemolysis process.

Hemolysis is recorded using a 96-well microplate reader (KRL instrument, Spiral, Dijon, France) by measuring the optical density (OD) decay at 450 nm. For each well, absorbance measurements are performed 75 times, once every 150 seconds. Results was expressed as the time required to reach 50% of maximal hemolysis (half-hemolysis time - HT50 - in minutes), which refers to the whole blood resistance to free-radical attack (Fig.5).

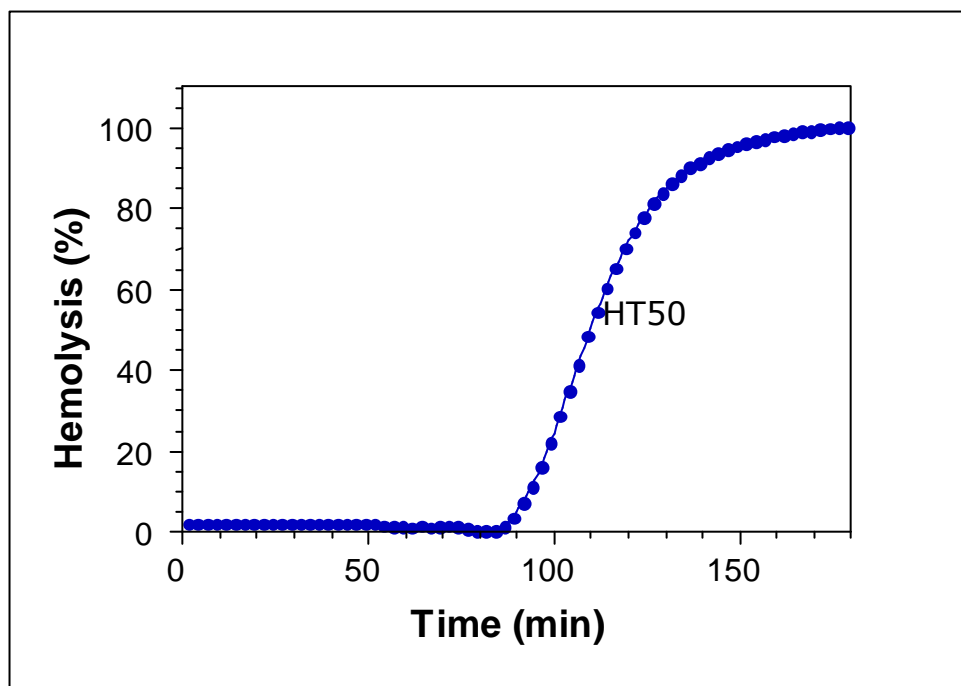


Fig.5. Percentage of hemolysis versus time.

Performance of KRL instrument, provided by the company, indicates a CV of the repeatability less than 2.5% and of reproducibility less than 4% (Laboratoires Spiral, France). Linearity range (tested with Trolox) is 5-1000 μl ; sensibility is 0.50 min/ μM (expressed by the slope of the straight line: $\text{HT50} = f([\text{Trolox}]\mu\text{M})$). Revelation limit is $< 1 \mu\text{M}$ eq. of Trolox, quantification limit is $< 5 \mu\text{M}$ eq. of Trolox.

Maximum speed of absorbance decrease (V_{max} in mUA/min), lag-time (in min) and HT50 (in min) are the variables of hemolysis kinetics calculated by KRL software (Laboratoires Spiral, France) (Fig.6).

Half-hemolysis time (HT50): time in which 50% of RBCs have been hemolyzed;

Maximum speed of absorbance decrease (V_{max}): the slope of the curve $\text{OD} = f(\text{time})$ at HT50;

Lag-time: time in which hemolysis begins.

Since hemolysis times vary in a linear way with the Trolox concentration, it is possible to convert them in EA (antiradical efficiency) where 1 unit of AE/L of blood or RBCs corresponds to the antiradical power of 1 mMol of Trolox /L of reference blood.

$$EAR_{(\text{blood or RBCs})} = HT50_{(\text{blood or RBCs})} / \Delta HT50_{(STD)} \times [Trolox] \times \text{dilution}_{(\text{blood or RBCs})}$$

where

$$\Delta HT50_{(STD)} = HT50_{(\text{control sample} + Trolox)} - HT50_{(\text{control sample})}$$

$$[Trolox] = 50 \text{mM}$$

$$\text{dilution}_{(\text{blood or RBCs})} = 135.$$

Sample	Vmax (mAU/min)	Lag-time (min)	HT50 (min)	E.A.R. eq. mM	T ₀ OD (AU)	ID subject	matrix	well
1	-24.80	70.76	90.32	30.18	0.822	piglet F 45d	Blood	A1
2	-28.00	72.76	90.56	30.26	0.845	piglet F 45d	Blood	B1
3	-27.60	72.70	90.12	30.11	0.840	piglet F 45d	Blood	C1
4	-40.40	65.04	77.15	25.78	0.856	piglet F 45d	Blood	D1
1	-42.40	44.58	55.94	18.69	0.835	piglet F 45d	RBC	E1
2	-32.40	51.89	64.50	21.55	0.768	piglet F 45d	RBC	F1
3	-34.80	53.95	67.22	22.46	0.818	piglet F 45d	RBC	G1
4	-6.80	38.85	48.02	16.05	0.134	piglet F 45d	RBC	H1

Fig.6. Example of KRL software output table.

Reference values have been calculated firstly for humans on a sample of healthy subjects (blood donors free from evident pathologies).

By mean of a multivariate statistic study, several risk factors, that lead to a decrease of antiradical defences, have been determined, such as age, sex, stress, sport activity, smoking, assumption of alcohol, vitamins and fruit.

The study allowed to define the population at risk, reference values have been calculated comparing healthy an at risk population.

A “normal” range has been determined considering the interval between the 14th (84 min) and the 86th (101 min) percentile (Fig.7); the range includes the 72% of the healthy subjects (specificity) and excludes the 70% of at risk subjects (sensitivity).

Reference value for human whole blood samples:

HT50 = 84-101 min, Lag time = 70-87 min.

Reference value for RBCs suspension (1:50):

HT50 = 66-75 min, Lag time = 52-61 min.

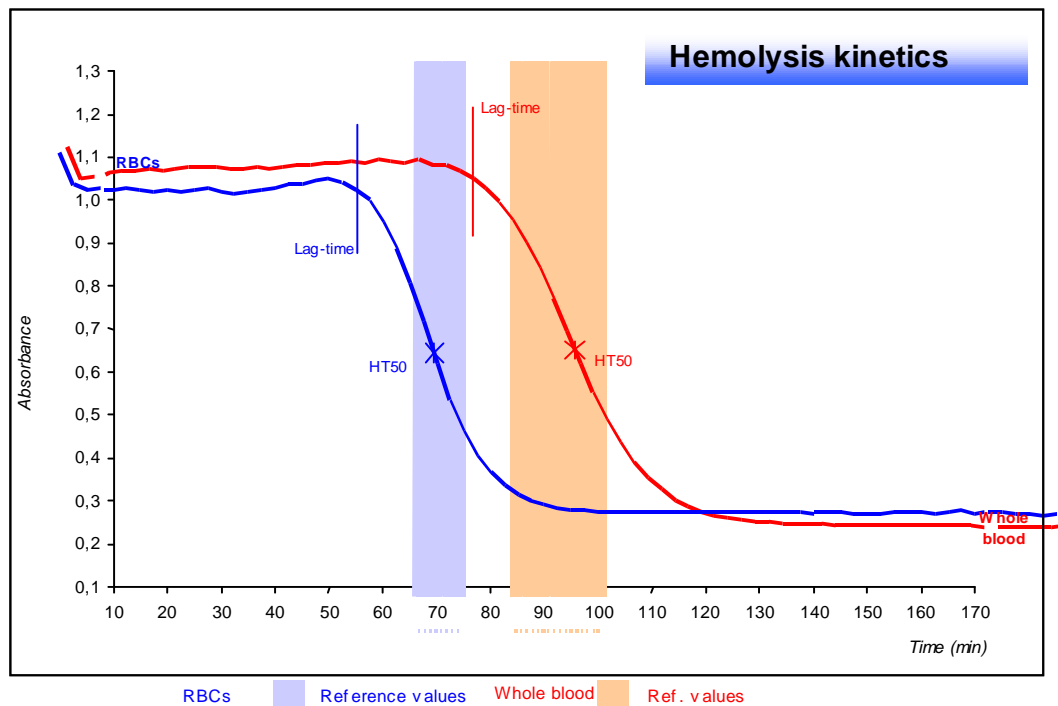


Fig.7. Example of KRL software output graphic.

Application of KRL

KRL test has been used to assess the effect of several factors on antioxidant defences in humans. As follows are reported results of some performed studies (Fig.8, Fig.9, Fig.10, Fig.11 and Fig.12).

Fig 8 - The effect of age and vitamin intake (Girodon et al., 1997).

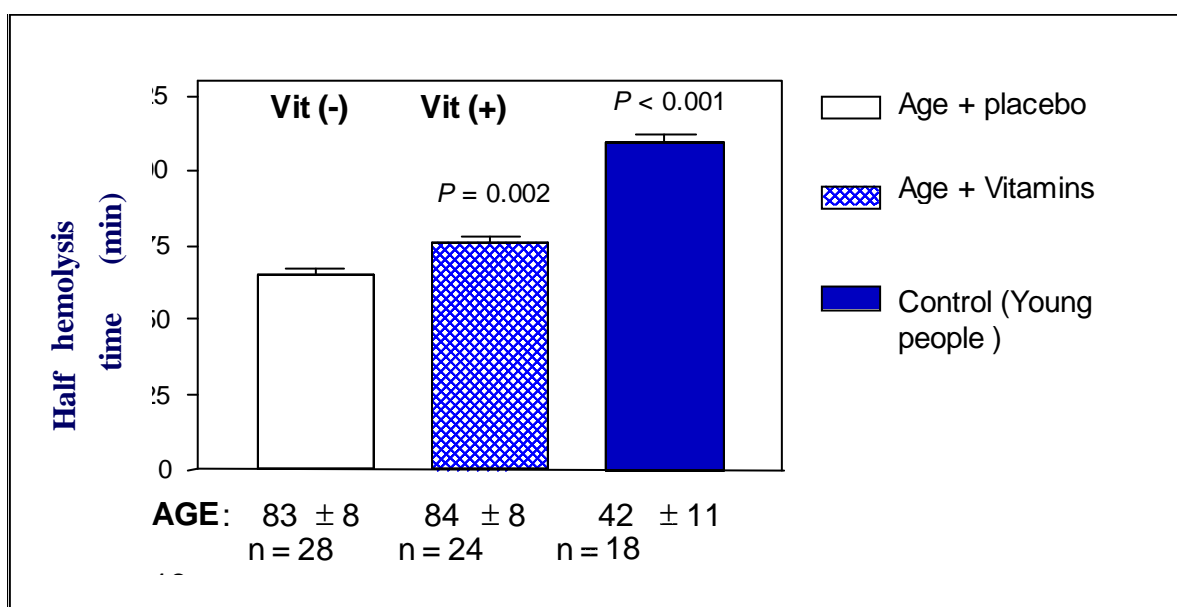


Fig 9 - The effects of lifestyle on the overall antioxidant capacity of healthy subjects (Lesgards et al., 2002).

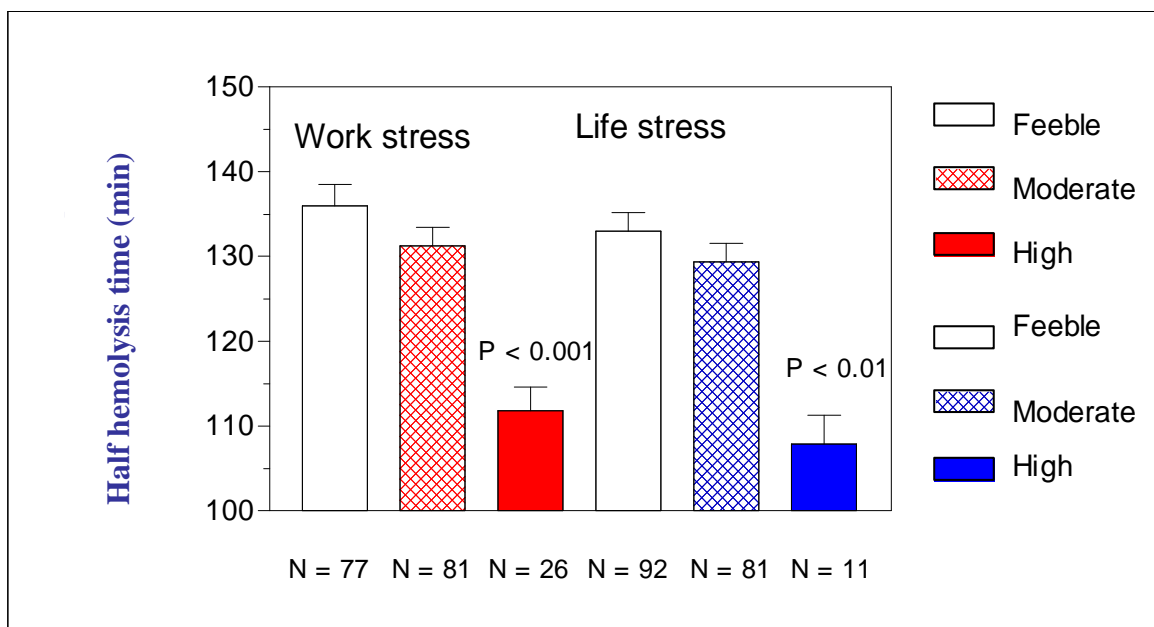


Fig.10 - Effect of sun exposition on global antiradical defense (Lesgards et al., 2002).

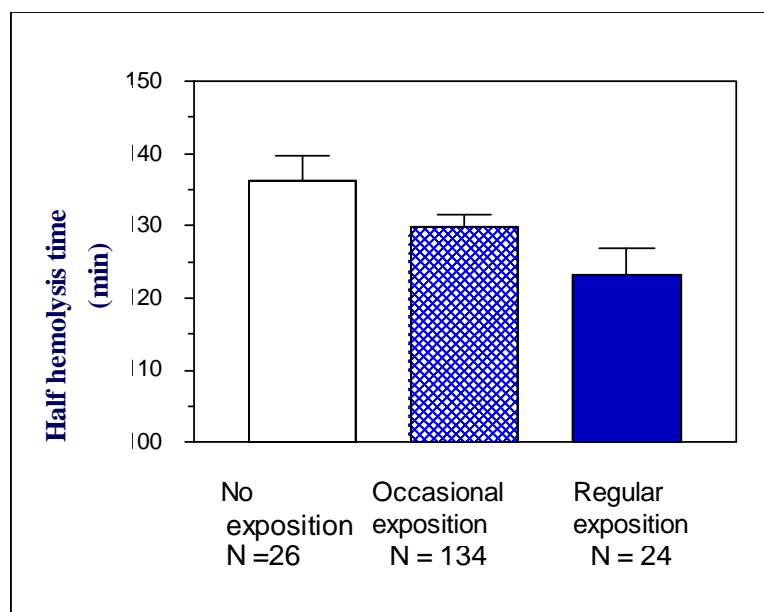


Fig.11 - Effect of vitamin supplementation and fruit consumption (Lesgards et al., 2002).

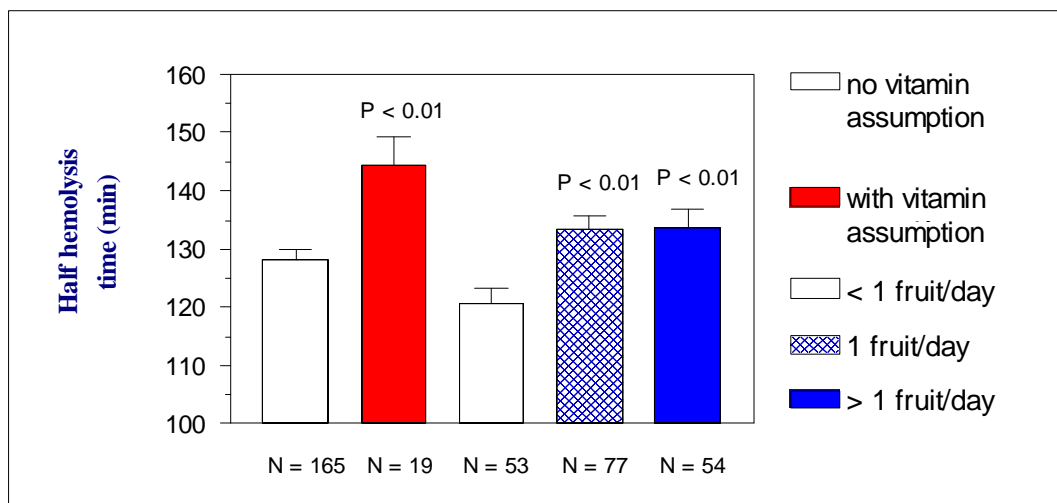
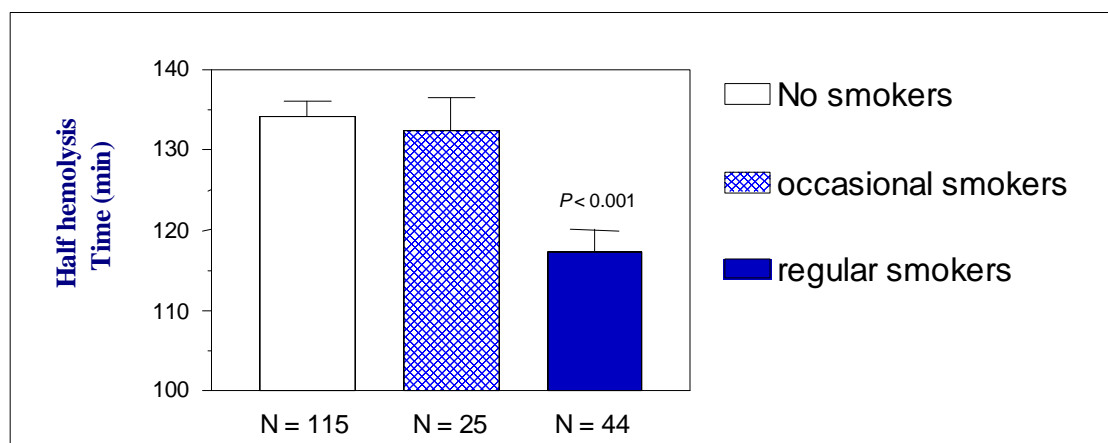


Fig.12 - Effect of tobacco smoking on global antiradical defense (Lesgards et al., 2002).



KRL test has been also used to test antiradical activity of different antioxidant substances in vitro; the addition of an antioxidant causes an elongation of the Lag time as showed in Fig.13, Fig.14, Fig.15.

Fig.13 - Uric acid :

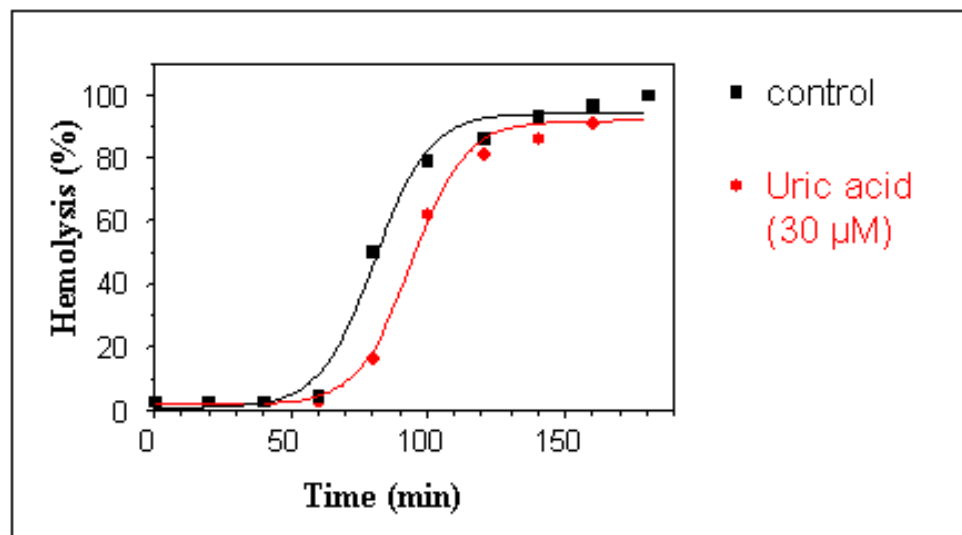


Fig.14 - Different concentration of vitamin C:

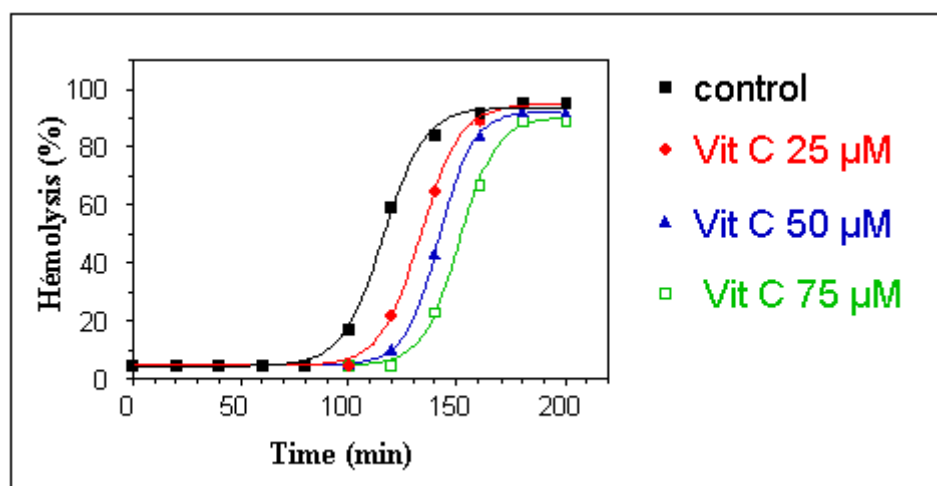
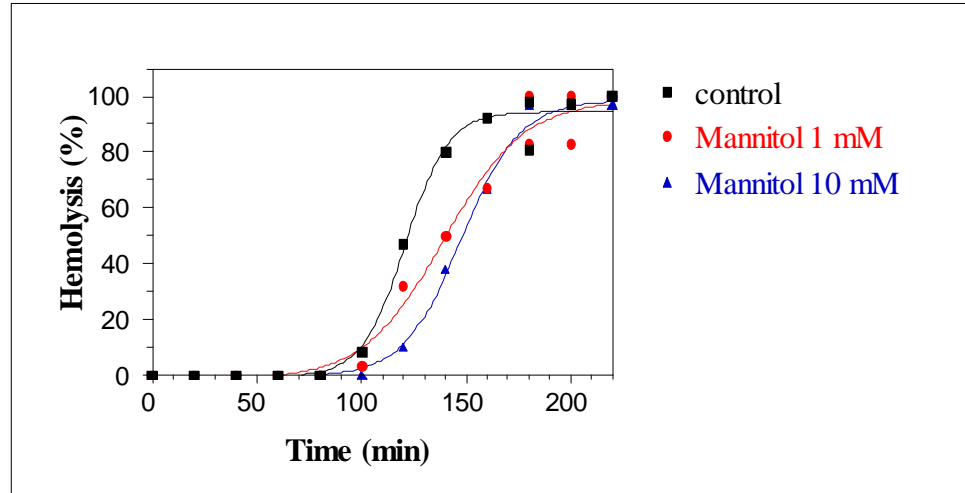


Fig.15 - Mannitol:



KRL test can be applied on animal sample in order to evaluate antioxidant defences of different species (wild animals or livestock) in different life conditions (different breeding systems).

Some studies on antioxidant defenses of animals, measured with KRL test, have been published, particularly on fishes (Bertrand et al., 2006), rats (Girards et al., 2005; Taleb-Senouci et al., 2009) and birds (Alvarez et al., 2006) but lacking are studies on animals reared in intensive farming.

CHAPTER 2

Objectives

2. Oxidative stress and animal welfare

Animal welfare is object of growing attention not only on the part of the scientific community but also of consumers, breeders and food producers. Several authors proposed different definitions of “animal welfare” trying to consider both ethical and scientific aspects.

Welfare is clearly a characteristic of an individual animal and is concerned with the effects of all aspects of its genotype and environment on the individual (Duncan 1981). Broom (1991) defines it as follows: the welfare of an animal is its state as regards its attempts to cope with its environment. Welfare therefore includes the extent of failure to cope, which may lead to disease and injury, but also ease of coping or difficulty in coping. An important part of this state is that which involves attempts to cope with pathology, i.e. the health of the animal, and this has important welfare implications. Furthermore, welfare includes pleasurable mental states and unpleasant states such as pain, fear and frustration (Duncan 1996, Fraser and Duncan 1998). Feelings are a part of many mechanisms for attempting to cope with good and bad aspects of life and most feelings must have evolved because of their beneficial effects (Broom 1998). Although feelings cannot be measured directly, their existence may be deduced from measures of physiology, behaviour, pathological conditions, etc. Good welfare can occur provided the individual is able to adapt to or cope with the constraints to which it is exposed. Hence, welfare varies from very poor to very good and can be scientifically assessed. The word stress is used by some authors when there is failure to cope or, similarly, when a pre-pathological state ensues, but others use it for any situation in which an organism is forced to respond to environmental challenge (EFSA, 2005).

The concept of welfare varying over a range and being measurable using a variety of indicators. One of the best estimate of biological fitness of animals in a housing or management system is lifetime reproductive success. Impaired reproductive success is indicated by delayed onset of reproduction during development, lengthened intervals between successive breedings, reduced litter size, and early death. A clear reduction in reproductive success in controlled conditions indicates poorer welfare (Broom, 1991).

Disease level is of considerable importance in welfare assessment because the welfare of diseased animals is almost always poorer than that of healthy animals. Susceptibility to disease is also an important indicator of welfare. If animals are kept in such a way that their immune systems are less effective in combating disease, there is clearly some inadequacy in the management and housing system (Fraser and Broom, 1990). One reason for impaired immune system function is

too frequent high activity of the adrenal cortex. Adrenal activity can occur during beneficial activities such as mating, but in general it indicates that the animal has some difficulty in trying to cope, so measurements of levels of adrenal products or of the activities of adrenal enzymes are useful welfare indicators. Behaviour is changed in response to many environmental difficulties. It is a component of both regulatory and emergency responses. Some measurements of behavioural responses to difficulties are of actions that help the animal to cope, whereas others are of behaviour pathologies that may have no beneficial effect. Both kinds of behaviour are indicators of welfare. Although one measurement can indicate that an individual is having severe difficulties in coping with conditions, it is essential that a variety of welfare indicators be used if an adequate assessment of animal housing and management systems is to be obtained (Broom, 1991).

2.1 Pig welfare

Societal concerns about conventional pig production have been increasing for a number of years in Europe. The conventional production system is generally thought to be associated with a negative environmental impact (pollution, offensive odours), and poor animal welfare due to high animal densities and bad housing conditions, and is perceived to result in reduced meat quality (Lebret et al., 2006). In intensive husbandry, pigs are often exposed to stressors such as mixing with unfamiliar animals, inconsistent handling procedures and exposure to new environments. Coping with these stressors can affect the welfare and production of pigs. One of the main issues in intensive farming practices is "overcrowding", which may lead to both sanitary and behavioural problems. Limited space availability can have detrimental effects on pigs: first of all, it may increase the frequency of agonistic interactions (Ewbank and Bryant, 1972). Similarly, Madsen et al. (1976) showed an increase of the occurrence of tail-biting in response to higher stocking density. Welfare is also affected by several environmental variables inside of housing system, or by action or relationship of climate parameters (temperature, humidity, air movement). When the environmental condition are good, the animal is able to show their better performances, allowing the maximization of farm economical results. Among these conditions, air quality (particularly ammonia) is an important indicator. In addition, in pig meat production over the last 50 years, genotypes have been intensively selected for fat reduction and fast growth in order to improve feed conversion. Although such selection criteria can lead to increased productivity and profitability, they may be detrimental to animal welfare (Brambilla et al., 2002).

From a methodological point of view, pig welfare can be assessed through three different approaches.

The first approach is based on determination of the subjective feelings of pigs, it points out animals' capacity to feel affective and emotional states. In this case ethological and physiological indicators are used.

The second approach is "functional", it is based on measures of objective welfare parameters provided by some physiological, pathological, productive and behavioural indicators: the normal functioning of biological systems of animals associated with good production performance is considered in itself an indicator of a satisfactory welfare condition.

Finally, the "natural" approach is based instead on the assumption that pigs in good welfare condition are able to manifest fully their behavioural repertoire.

Evaluating pig welfare through the functional approach, physiological indicators most frequently used are: heart and respiratory rate, glycemia, cholesterol, total blood proteins, glucocorticoids, and oxidative stress; immunological indicators most used are: serum antibacterial activity, haptoglobin and lysozyme. There's a clear correlation between welfare and immunological status of pigs. Cortisol release due to stress stimuli causes a decrease in the number of lymphocytes and a concomitant increase of granulocytes and neutrophils (Schouten, 2001). Pathological indicators most frequently used are: poor growth, gastric ulcers incidence, immunosuppression, ipofertility and excessive mortality rate, most utilised productive indicators are: weight gain, food conversion and disease resistance.

An animal welfare assessment that takes into account the behaviour and activities of animals is important but does not allow to highlight the initial conditions of physiological imbalances that can lead to serious illness or disease status. According to the functional approach, initial conditions of physiological imbalances could be identified through the assessment of oxidative stress. Brambilla et al. (2002) have considered the response to oxidative stress an effective parameter for assessment of welfare in pigs.

Due to a genetic susceptibility, rapidly growing feeder pigs are quite exposed to oxidative stress; the indiscriminate over-selection for single traits in pigs has caused many problems in lean, fast growing pigs. In some in-bred lines, the possible deficient oxygen supply in tissues can determine the systematic release, within distinct cell compartments, of reactive oxygen species, such as superoxide anions, nitrogen oxide and hydroxyl radicals (Brambilla et al., 2001; Brambilla et al., 2002). Such free radicals are involved in the pathogenetic mechanism of the following metabolic diseases: Mulberry heart disease (MHD), which is caused by extensive oxidative-based cardio-angiopathy; Porcine stress syndrome, characterised by an abnormal accumulation of lactic acid in the muscle cells; and

osteochondrosis, which is due to altered bone-growth metabolism (Brambilla and Cantafora, 2004).

Brambilla et al. (2002) used two biochemical assays based on the Fenton reaction able to trace the early state of oxidative stress at farm level. The first method is the ROMs test (described previously), the second one determines the amount of free radical scavengers in animal sera (Oxy assay). The assays provided objective biochemical data that allowed to assess the coping ability of farmed pigs, demonstrating that methods that assess total antiradical capacity can be valid instruments to quantify oxidative stress in swine.

In the present thesis I will analyze the response to stress measured with KRL test in post weaning piglets (experimental trial 1, 2 and 3), in growing pigs fed with a diet supplemented with natural antioxidants (experimental trial 4) and in growing pigs reared in different floor types (experimental trial 5).

2.1.1 Weaning and stress physiology in pigs

Weaning has been defined as the time of final cessation of nursing and suckling activities (Counsilman and Lim, 1985). More generally, weaning marks the end of the preferential relationships between the sow and the piglets. The rupture of the maternal link for milk supply has consequences on gut local immune status of the piglet and on the gut microflora (Barnett et al., 1989; Hampson et al., 1985). *E. coli* bacteria can take advantage of the disturbed situation in the biotype to proliferate and produce toxins. Finally, changing of accommodation and mixing usually occur at weaning. The obvious consequence of all these simultaneous physical, nutritional, immunological and emotional changes is that weaning starts a critical phase (Pajor et al., 1991). Therefore, the weaning transition is a complex period during which piglets have to adapt rapidly to major changes in their nutrition and environment. Weaning at an early age (21–28 d), as in European intensive production systems, probably exacerbates the level of general stress in these immature animals.

The integration of piglet feeding with natural or not antioxidant such as phenylpropanoid or Vitamin E would be expected to have an antioxidant and immunological effect able to contain stress, measured as total radical activity, in this critical period.

2.1.2 Floor type and stress physiology in pigs

There is almost no published literature on stress physiology of pigs in relation to floor type. In a masters thesis (von Borrell, 1984) it was reported that the cortisol concentration of weaned piglets kept in flat decks did not differ from that of pigs housed in a reference system (0.5 m² per pig on solid floor with straw). In a

comparison of different floor materials and space allowances, pigs raised on iron slats at 0.23 m² per pig had the highest cortisol, whilst those raised on plastic coated floors at 0.30 m² per pig had the lowest level (pigs on plastic slats at 0.23 m² per pig and cast iron floors at 0.45 m² per pig showed intermediate values).

In an unreplicated group investigation, growing pigs of 35-90 kg, housed on an unbedded solid floor at an allowance of 0.5 m²/ pig, showed elevated plasma cortisol level in comparison with pigs given straw bedding when sampled by venepuncture approximately every two weeks over a 10 week trial period (Warnier and Zayan, 1985).

There have been physiological studies in which space allowance and floor type have been confounded. De Jong et al. (1998) compared growing pigs in fully-slatted pens with others given straw bedding and 140% (4-10 weeks) then 40% (10 weeks to slaughter at 27 weeks) higher space allowance. The pigs during the suckling phase had also previously been in conditions which differed in space and straw provision. When tested at 10 weeks of age, pigs from enriched housing had significantly higher baseline cortisol. Although higher baseline cortisol levels are often associated with chronic stress, the authors concluded that this was not an appropriate interpretation for the findings on enriched pigs in this study because of the lack of difference in response to stressors .

They hypothesised that the difference in baseline cortisol might be due to a change in circadian pattern, and this was investigated further in a second experiment which again involved confounding of space allowance and straw provision (de Jong et al., 2000).

It is difficult to draw conclusions starting from published data; for this reason the trial relative to different floor types has been planned.

CHAPTER 3

Total antiradical activity in male castrated piglets blood: reference values

3. Total antiradical activity in male castrated piglets blood: reference values

3.1 Abstract

Blood samples from 146 male castrated piglets in the range of 10 - 47 kg body weight were collected from the same farm and analysed for total antiradical activity in order to determine reference intervals. Data were tested for normality and then submitted to reference limit evaluation. The reference values found in piglets, expressed as half-hemolysis time (59.34 – 93.60 and 43.94 – 66.90 minutes for blood and red blood cell, respectively), are lower than those found in humans; further studies are needed to extend reference values study to female and to animals of different weight classes and different genetic type.

3.2 Introduction

Animal welfare is of concern in order to enhance animal performance and meet the request of consumers for quality and safety of products (Blokhuis et al., 2003). Metabolic, endocrine and immunity parameters are known as welfare indicators, expressing adaptation to the environment. The measure of total antiradical capacity of blood is used in humans as indicator of stress, and can be assessed through KRL test (Prost, 1989, 1992). KRL is a biological test which measures the time needed to hemolyze 50% of the red blood cells exposed to a controlled free radical attack. The principle of the biological test is to submit whole blood to a thermocontrolled free radical aggression in order to mobilize all families of free radical scavengers present in the blood to fight off the oxidant attack (Stocker et al., 2003; Pieri et al., 1996; Girodon et al., 1997). The KRL test have several applications in vivo, particularly tested on human studies, or in vitro. In humans KRL is used to study the effectiveness of natural or pharmaceutical treatments or to discover acute processes such as trauma and ischemia or inflammatory disease (Lesgards et al., 2002); moreover it allows to discriminate welfare conditions depending on medium or high stress, or tobacco smoking. According with Brambilla et al., (2002) the response to oxidative stress could be considered as welfare parameter in swine. As a consequence, firstly there is the necessity to establish reference values for KRL test in swine. The aim of the study is to establish blood and red blood cell (RBC) reference values for male castrated piglet in post weaning period.

3.3 Materials and methods

One hundred and forty six Goland hybrids piglets (all castrated males) in the same range of liveweight (10-30 kg) were selected from the same farm. All piglets were healthy. Blood was drawn by jugular into tubes containing EDTA. All samples were immediately forwarded to the laboratory at a temperature of 6°C and submitted to analysis within 24 h of collection.

Total antiradical potential for each piglet was evaluated by using a biologic test based on free-radical-induced hemolysis (Laboratoires Spiral, France) (Prost, 1989; Blache and Prost 1992). Whole Blood and RBC samples diluted to 1/50 was submitted in isotonic saline solution to organic free radicals produced at 37°C under air atmosphere from the thermal decomposition of a 27 mmol/L solution of 2,2'-azobis (2-amidinopropane) dihydrochloride (Spiral, Dijon, France).

Hemolysis was recorded using a 96-well microplate reader by measuring the optical density decay at 450 nm. Results was expressed as the time required to reach 50% of maximal hemolysis (half-hemolysis time - HT₅₀ - in minutes), which refers to the whole blood resistance to free-radical attack. Performance of KRL instrument provided by the company, indicate a CV of the repeatability less than 2.5% and of reproducibility less than 4% (Laboratoires Spiral, France). Since haemolysis times vary in a linear way with the trolox concentration, it is possible to convert them in AE (antiradical efficiency) where 1 unit of AE/L of blood or RBC corresponds to the antiradical power of 1 mMol of Trolox /L of reference blood.

All data were processed to obtain descriptive statistics and tested for normality by a Kolmogorov-Smirnov test showing Gaussian distribution ($P > 0.001$) and by graphics (Figure 1, 2). Reference values were calculated as mean \pm 1.96 SD, where SD is the standard deviation from the mean (Farver 1997).

3.4 Results and Conclusion

Univariate descriptive statistics are summarized in table 1 and reference values reported in table 2. Average HT₅₀ expressed as minutes found in male castrated pigs showed the blood values was higher than RBC (table 1) according to the different antioxidant system present in whole blood or in red blood cell analysed; in fact in whole blood, either extracellular or intracellular antioxidant defenses contribute to maintaining blood cell membrane integrity and function until cell lysis (Monaghan et al., 2009).

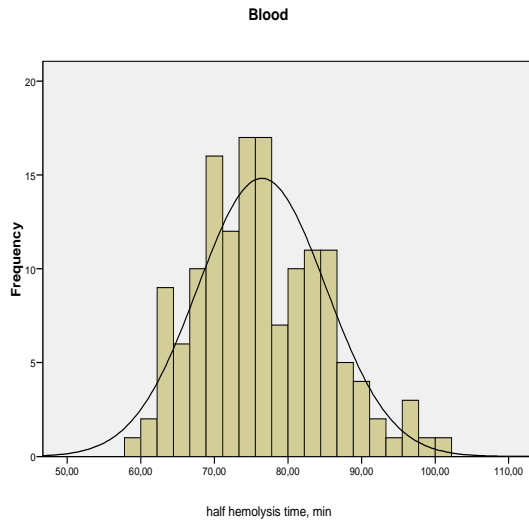


Figure 1
Frequency distribution of half-hemolysis time in blood

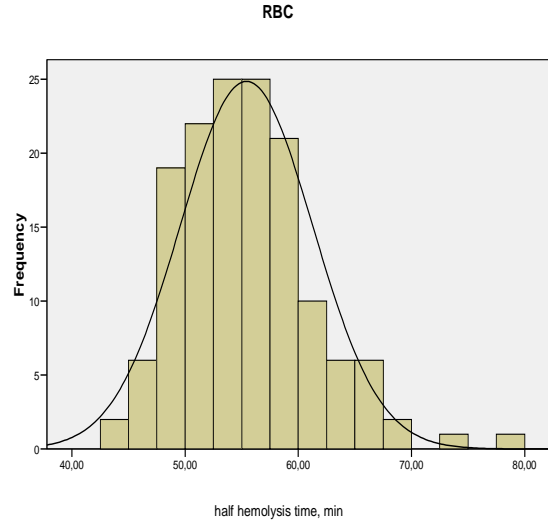


Figure 2
Frequency distribution of half hemolysis time (min) (min) in Red Blood Cell (RBC)

Table 1. Descriptive statistics of age, body weight (BW) and overall antioxidant capacity, evaluated as half-hemolysis time in minutes (HT_{50}), in castrated male piglets (N=146)

	Mean \pm SE	CV ¹ %	Median	Mode
Age, days	69 \pm 1	29.9	58	58
BW, kg	22 \pm 0.9	47.0	20.1	29.00
HT_{50} , min				
- Blood	76.46 \pm 0.72	11.41	75.58	76.59
- RBC ²	55.42 \pm 0.48	10.57	54.98	58.06

¹ Coefficient of variation

² Red Blood Cell

Since the measurement of HT_{50} is very reproducible KRL test has been shown to be representative of the overall defense against free radicals in humans and animal models (Prost, 1989, Girodon et al., 1997, Bourdon 1999).

Table 2. Reference values of the overall antioxidant capacity, expressed either as half-hemolysis time in minutes (HT_{50}) either as antiradical efficiency (AE), in castrated male piglets.

	HT_{50} , min	AE eq mMol
Blood, min-max	59.34 – 93.60	19.82 – 31.28
Red blood cell (RBC), min-max	43.94 – 66.90	14.67 – 22.36

The reference values found in male piglets are lower than those found in humans (84 – 101 and 66 – 75 min respectively for blood and RBC). The lower reference values in piglets may be related to the age, the housing systems and likely, to differences among species. Values of HT_{50} assessed on blood or RBC have different interest for researchers. The analysis on blood allows to measure intracellular and extracellular defense considering the synergic effect of the two actions, whereas RBC analysis concerns especially the intracellular defense status. The two information are complementary. The RBC mean life in pig is 60-85 days, consequently RBC is important for the interpretation of the balance between attack and defence of organism in a medium/long period. On the contrary whole blood parameter gives an indication more immediate of the physiological status.

The parameters proposed could be useful especially for the evaluation of welfare in intensive pigs system for studying the influence of housing systems and nutrition. Further studies are needed to extend reference values to female and to animals of different weight classes, genetic types and with different feed rationing plane.

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CHAPTER 4

Effect of weaning on total antiradical activity in piglets

4. Effect of weaning on total antiradical activity in piglets

4.1 Abstract

The aim of the present study was to evaluate the weaning effect on the total antiradical activity in piglets. At weaning (24 day), forty castrated male piglets of an average live weight of 7.1 ± 0.8 kg, were randomly selected, moved to a pens (10 piglets per pen) and reared in an environmentally controlled room. Fasting blood samples were taken by all the subjects by anterior vena cava puncture at: weaning (T0), and at 15 (T1) and 60 (T2) days after weaning. The blood samples were immediately analyzed for the determination of the total antiradical activity, using the KRL biological test. Whole blood and red blood cells solutions were incubated at 37 °C for 15 min before being submitted to free radicals produced by a final 50 mM solution of AAPH. Haemolysis was recorded using a 96-well microplate reader by measuring the optical density decay at 450 nm (Laboratoires Spiral, France). Results, expressed as the time that is required to reach 50% of maximal haemolysis (half-haemolysis time, HT50 in minutes) which refers to the erythrocyte resistance to free radical attack. A mixed model ANOVA was used to assess the main effect of time and a random effect of animal on whole blood and red blood cells total antioxidant activity. The half-haemolysis time of whole blood declined ($P < 0.001$) from 102.8 ± 9.8 min in nursing piglets of 24 days to 86.4 ± 7.5 min and 73.4 ± 6.5 min at 15 and 60 days after weaning respectively. The same result was observed for the half-haemolysis time of red blood cells that decreased ($P < 0.001$) from 70.4 ± 9.8 min in nursing piglets of 24 days, to 59 ± 4.4 min and 53.5 ± 4.8 min at 15 and 60 days after weaning respectively. Overall these result indicate that total antioxidant activity in piglets blood significantly decline after weaning. This gives clear evidence that dietary antioxidant supplementation after weaning may positively affect the antioxidant status improving pig health.

4.2 Introduction

Oxidative stress occurs when the antioxidant system is overwhelmed by the production of reactive oxygen species, which can lead to increased prevalence of infectious disease via impaired immune cell function, and perhaps various sudden death syndromes. Two of the critical stages for dietary antioxidant vitamin E as a nutrient for growth and health status in pigs are immediately after birth and after weaning (Lauridsen et al., 2005). In fact as reported by Weiss et

al. (2008) mortality of weaned piglets was greatly reduced when they were injected with two important components of the antioxidant systems such as Se and/or vitamin E. The aim of the present study was to evaluate the effect of weaning on the total antiradical activity on whole blood and red blood cells in piglets, using the KRL test.

4.3 Materials and methods

At weaning (24 day), forty castrated male piglets, 7.1 ± 0.8 kg BW, were randomly selected, moved to a pens (10 piglets per pen) and reared in an environmentally controlled room. Fasting blood samples were taken by all the subjects by anterior vena cava puncture at: weaning (T0), and at 15 (T1) and 60 (T2) days after weaning. The blood samples were immediately analyzed for the determination of the total antiradical activity, using the KRL biological test (Prost, 1992), that allows the evaluation of red blood cell resistance against the free radicals induced by 2,2'-azobis (2-amidinopropane) hydrochloride (AAPH). Whole blood and red blood cells solutions were diluted to 1:50 in phosphate buffer in isotonic conditions at pH = 7.4 and were incubated at 37 °C for 15 min before being submitted to free radicals produced by a final 50 mM solution of AAPH. Haemolysis was recorded using a 96-well microplate reader by measuring the optical density decay at 450 nm (Laboratoires Spiral, France). Results, expressed as the time that is required to reach 50% of maximal haemolysis (half-haemolysis time, HT50 in minutes) which refers to the erythrocyte resistance to free radical attack. A mixed model ANOVA was used to assess the main effect of time and a random effect of animal on whole blood and red blood cells total antioxidant activity.

4.4 Results and discussion

The total antiradical activity, expressed as half-haemolysis time, in whole blood declined ($P < 0.001$) from 102.8 ± 9.8 min in nursing piglets to 86.4 ± 7.5 min and 73.4 ± 6.5 min at 15 and 60 days after weaning respectively (Figure 1).

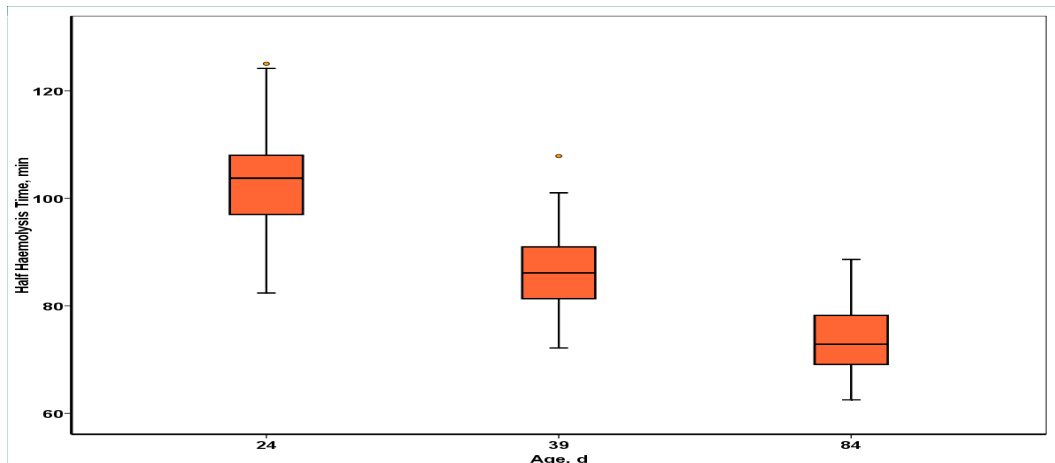


FIGURE 1. Total antiradical activity of piglets whole blood at weaning (24 d) and at 15 and 60 days after weaning

The same result was observed for the half-haemolysis time of red blood cells that decreased ($P < 0.001$) from 70.4 ± 9.8 min in nursing piglets to 59 ± 4.4 min and 53.5 ± 4.8 min at 15 and 60 days after weaning respectively (Figure 2).

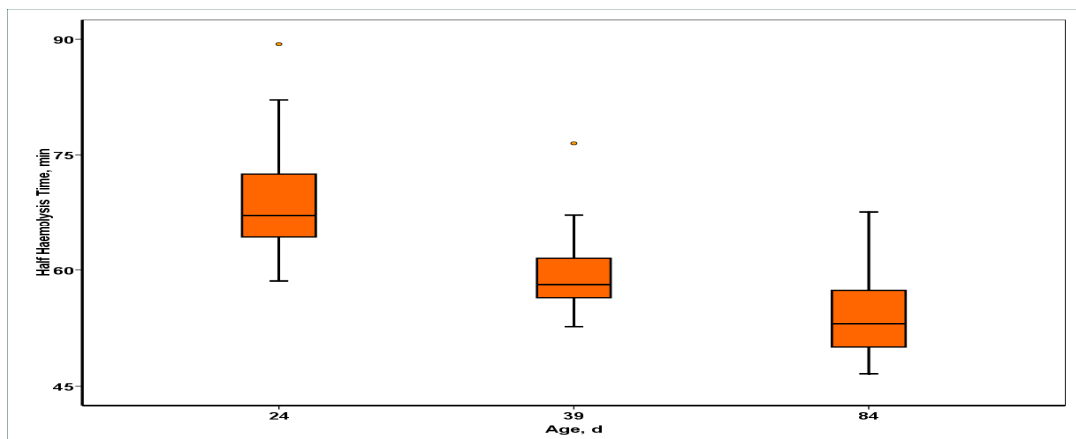


FIGURE 2. Total antiradical activity of piglets red blood cells at weaning (24 d) and at 15 and 60 days after weaning.

4.5 Conclusions

Overall these results indicate that total antioxidant activity in piglets whole blood and red blood cells significantly decline after weaning. Profitable swine production requires rapid increase of body weight and lean tissue, raising the demand on the metabolic system of these animals which can lead to increased oxidative stress unless antioxidant systems are enhanced via nutrition. In fact, as previously observed in weaned piglets, oxidative status and growth performances were improved in animals feeding natural integration with the phenylpropanoide

glycoside verbascoside (Corino et al., 2007). This gives clear evidence that an adequate antioxidant supplementation after weaning may positively affect piglets total antiradical activity and improve health.

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CHAPTER 5

Antioxidant supplementation in post weaning piglets: effects on total blood antiradical activity and serum haptoglobin

5. Antioxidant supplementation in post weaning piglets: effects on total blood antiradical activity and serum haptoglobin

5.1 Abstract

The aim of the present work was to evaluate the effect of antioxidant supplementation in post weaning piglets on total blood antiradical activity and serum haptoglobin. At weaning 80 piglets of an average weight of 7 kg were allotted within weight to two dietary treatments: control diet, contained 175 mg/kg of Vitamin E and a diet supplemented with the basal dosage plus 54 mg/kg of vitamin E. The animals were weighted at the beginning of the trial, at 15 and 60 d after weaning. Blood samples were taken randomly from 10 piglets per treatment at weaning, 15 and 60 d post weaning for the determination of total blood antiradical activity and serum haptoglobin. The determination of total blood antiradical activity was carried out by the biological test KRL. Serum concentrations of haptoglobin were measured using a commercial kit (Phase™ Range Haptoglobin Assay, Tridelta Development Ltd.). Growth performance and serum haptoglobin concentration did not differ between experimental groups. The total antiradical activity of whole blood and red blood cells resulted higher in piglets supplemented with vitamin E than control ($P < 0.05$). Supplementation of vitamin E may improve resistance to oxidative stress in post weaning piglet, enhancing animal welfare.

5.2 Introduction

Weaning is a critical phase for piglets, it is characterized by a decrease of growth performance, high incidence of diseases, immunodepression and increased mortality (Odle et al., 1996; Frydendahl, 2000). At weaning blood vitamin E concentration decreases to a critical level and this decline is often associated to onset of deficiency symptoms (Lauridsen and Jensen, 2005). Vitamin E plays a key role in immune system development (Jensen et al., 1988) and it reduces oxidative stress, that is particularly elevated in post-weaning piglets (Sauerwein et al., 2005). Oxidative stress assessment is an effective pig welfare indicator (Brambilla et al., 2002), it can be combined with immunological indicators, such as haptoglobin. Haptoglobin is an acute phase protein, it is an effective indicator of inflammation and serum haptoglobin can be used as an indicator of welfare in swine (Amory et al., 2007). The aim of this study is the evaluation of the

effects of a vitamin E enriched diet on welfare of post weaning piglets, using blood global antiradical activity and serum haptoglobin as indicators.

5.3 Materials and methods

80 piglets, half males and half female, weighing about seven kilograms of initial, were divided into two experimental groups, in a uniform manner according to live weight and sex. The animals were fed *ad libitum* throughout the experimental period with two isoenergetic and isoproteic diets. The control group (C) received a 175 mg/kg of vitamin E dose by day, while the treated group received a diet supplemented with the basal dosage plus 54 mg/kg of vitamin E. The trial had a duration of 60 days. The animals were weighted at the beginning of the trial, at 15 and 60 d after weaning for the calculation of ADG.

At the beginning of trial, at 15 and 60 d after weaning blood sampling was performed on 10 subjects per treatment. Whole blood samples were analysed for the determination of global antiradical activity of blood using the KRL test. The principle of the biological test is to submit whole blood to a thermocontrolled free radical aggression in order to mobilize all families of free radical scavengers present in the blood to fight off the oxidant attack (Prost,1992). Whole Blood and red blood cells (RBCs) samples diluted to 1/50 was submitted in isotonic saline solution to organic free radicals produced at 37°C under air atmosphere from the thermal decomposition of a solution of 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) (Kirial International, Dijon, France). Hemolysis was recorded using a 96-well microplate reader by measuring the optical density decay at 450 nm. For each well, absorbance measurements are performed 75 times, once every 150 seconds. Results was expressed as the time required to reach 50% of maximal hemolysis (half-hemolysis time - HT50 - in minutes), which refers to the whole blood resistance to free-radical attack. A commercial kit (Phase™ Range Haptoglobin Assay, Tridelta Development Ltd.) was used to determine serum haptoglobin. Data on growth performance were subjected to analysis of variance (ANOVA). The results for the blood parameters were analyzed by ANOVA for repeated measures with the weaning value entered as a covariate.

5.4 Results

Data on growth performance did not show differences between the two groups. The average weight of animals taken at the beginning of the trial was 7.2 kg, 9.5 kg at 15d and 28.6 kg at 60 d. The ADG was 414 g/d. Whole blood and RBCs antiradical activity is reported in Fig.1. Whole blood and RBCs antiradical

activity is significantly higher ($P < 0.05$) in the treated group compared to control. No effect of time of sampling or duration of treatment was detected.

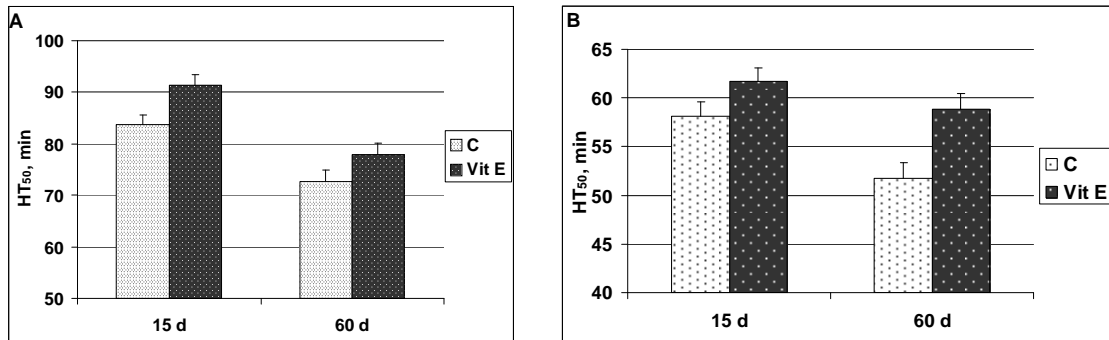


Fig. 1. Whole blood (A) and RBCs (B) antiradical activity of treated and control piglets at 15 and 60 d after weaning.

Serum haptoglobin did not show significant differences in treated and control groups at different time of sampling, as reported in Figure 2.

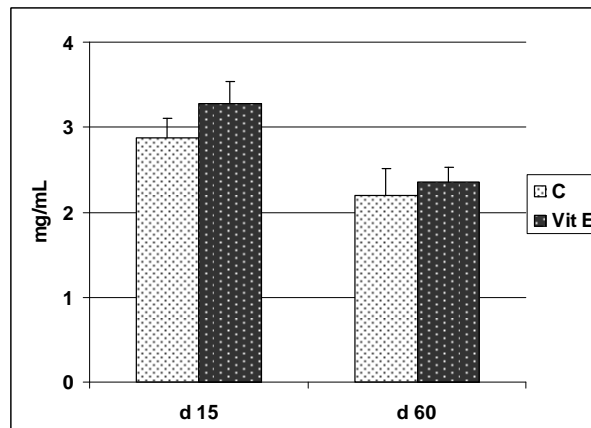


Fig. 2. Serum haptoglobin of treated and control piglets at 15 and 60 d after weaning.

5.5 Conclusions

The administration of different levels of vitamin E showed no improvement in the growth performance of post weaning piglets, in agreement with the literature (Chung et al., 1992; Wilburn et al., 2008). Corino et al. (2007) by contrast, reported positive effects on growth performance and blood oxidative status of post-weaning piglets fed with a diet supplemented with natural antioxidants. The higher antiradical activity of whole blood and red blood cells, as evidenced in the group supplemented with vitamin E, indicate increased resistance of animals to oxidative stress. This could lead to a lower occurrence of diseases related to

oxidative stress, such as enteritis, pneumonia and sepsis (Lykkesfeldt and Svendsen, 2007). The level of vitamin E used in this study was not able to reduce the concentration of serum haptoglobin, according to Møller and Lauridsen (2006) reported that as the integration of the diet with 300 mg / kg of vitamin E did not bring to reduced production of inflammatory markers such as cytokines and eicosanoids. In conclusion, supplementation with vitamin E appears to positively counteract oxidative stress in post-weaning piglet in facilitating the passing of one of the most critical phases of pig farming. The KRL test was able to highlight, in a statistically significant way, the difference between the two experimental groups that neither growth performance nor haptoglobin were able to detect.

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CHAPTER 6

Use of Phenylpropanoids in pig diet: effects on total blood antiradical activity and some serum biochemical parameters

6. Use of Phenylpropanoids in pig diet: effects on total blood antiradical activity and some serum biochemical parameters

6.1 Abstract

The aim of the present work was to evaluate the effect of natural antioxidant supplementation in pigs from weaning to 97 kg LW on performance and on some physiological indicators of welfare. Forty piglets of 7 kg LW were allotted to two dietary treatments: control diet and supplemented diet (5 mg/kg of a natural extracts titered in phenylpropanoid glycosides (PPG) expressed as verbascoside). Pigs were weighted at d 0, d 15, d 60, d 90 and d 150 after weaning. Blood samples from 12 piglets/treatment were analysed for serum haptoglobin, cortisol, aspartate amino transferase (AST) and bilirubin concentrations and blood antiradical activity by mean KRL test. No differences were found for performance. Total antiradical activity tended to improve in treated group. Haptoglobin and AST showed time effect ($P < 0.001$).

6.2 Introduction

The European Union, in January 2006 banning the use of any auxinic substances for the prophylaxis of diseases in livestock, has moved the research into the possible use in animal feed of natural extracts that can influence the performance and improve the well-being. Among natural substances, the phenylpropanoid possess strong antioxidant properties that can counteract oxidative stress. Over the past 20 years there has been an increasing interest in the biology of free radicals (Machlin and Bendich, 1987). Free radicals are compounds characterized by an unbalanced electronic structure that gives them a great reactivity on organic constituents and cellular structures. Under normal conditions the production of free radicals in an organism is balanced by a number of protective factors. Free radicals exert their negative effect at cellular level: peroxidation of polyunsaturated fatty acids of phospholipid membranes leads to production of cytotoxic peroxides that cause inflammation and cell death. In addition, the oxygen radical intermediates are activators of carcinogenesis and cardiovascular diseases. The measure of the total antiradical capacity of blood, used in the human as an indicator of stress, (Lesgards et al., 2002) can be determined through the KRL test (Kit free radicals) (Prost, 1989, 1992).

The objective of this study was therefore to assess the effects of diet supplementation, in post weaning pigs and in growing pigs up around 100 kg LW, on growth performance and on some blood parameters often used as physiological indicators of welfare .

6.3 Materials and methods

For the experimental trial 40 castrated male pigs of initial weight of about 7 kg were used, divided into two experimental groups of 20 subjects each. The animals were marked individually by ear tag and uniformly distributed on the basis of live weight in two different experimental groups for integration in the feed with a natural extract titrated in phenylpropanoid glycosides (PPG), adding 5 mg of PPG, expressed as verbascoside, per kg of feed. The animals were fed *ad libitum* throughout the experimental period of 150 days with isoenergetic and isoproteic diets. During the test, were found the weight of pigs at 0, 15, 60, 90 and 150 days, the average food consumption of the box, the mortality rate and therapeutic interventions; at the weighing were also collected 5 blood samples from 12 subjects per group for the analysis of the following parameters: haptoglobin, using a commercial kit (Phase™ Range Haptoglobin Assay, Tridelta Development Ltd), serum cortisol (using the automatic analyzer IMMULITE ONE, Medical System, Genova, Italy and the commercial kit LKC01; Medical System, Genova, Italy), bilirubin (using the biochemical analyzer Roche BM Hitachi 911 kit Roche BIL-T) and AST (using the biochemical analyzer Roche BM Hitachi 911 kit Roche AST). These parameters' concentration increases in response to problems of infection, inflammation, trauma, immune disorders, cancers (haptoglobin), or the toxic, infectious and inflammatory events (AST), or specific diseases (bilirubin), or various types of stress (cortisol) . The evaluation of oxidative stability on whole blood or red blood cells was measured by a biological test to measure the global antiradical defense (KRL). This test requires that blood samples and/or red blood cells, diluted with isotonic solution 1/50 and 1/25, respectively for the red blood cells and whole blood, are subjected to a chemical stress induced by the addition of a generator of free radicals represented by 2,2'-azobis (2-amidinopropane) dihydrochloride (Spiral, Dijon, France). The test measures the decrease in absorbance corresponding to the progressive disappearance of cells; the resistance of blood or red blood cells to attack by free radicals is expressed through the time required for 50% lysis of blood cells (T1/2). The results were evaluated with the Student test. Data on blood tests were subjected to analysis by ANOVA for repeated measures with the weaning value entered as covariate.

6.4 Results

The growth performance showed no significant differences between groups. The average final weight of the animals stood about 97 kg, with an average daily gain of 598 g/d. The feed conversion showed no significant differences in the whole period and it was found to be 2.4 and 2.5 in the control and treated group respectively. The therapeutic treatments and mortality showed no differences between the two groups. The results for the biochemical parameters are presented in Table 1.

	Trattamento		Tratt	P	
	Controllo	PPG		T	T*Tratt
Aptoglobina, mg/mL					
15 d	2.1 ± 0.4	2.4 ± 0.4			
60 d	1.9 ± 0.3	2.1 ± 0.3			
90 d	1.3 ± 0.3	1.3 ± 0.2			
150 d	0.4 ± 0.1	0.6 ± 0.1			
			0.403	<0.001	0.948
Cortisolo, µg/dL					
15 d	3.9 ± 0.4	3.7 ± 0.4			
60 d	2.5 ± 0.3	2.3 ± 0.3			
90 d 15	2.3 ± 0.2	2.1 ± 0.2			
150 d	1.7 ± 0.2	1.6 ± 0.2			
			0.553	0.286	0.996
Bilirubina, µmoli/L					
15 d	4.6 ± 0.5	4.1 ± 0.5			
60 d	5.1 ± 0.4	4.2 ± 0.4			
90 d	3.0 ± 0.2	3.9 ± 0.3			
150 d	4.7 ± 0.6	4.5 ± 0.6			
			0.637	0.850	0.256
Aspartato amino transferasi, UI/L					
15 d	54.0 ± 3.7	41.3 ± 3.2			
60 d	40.9 ± 2.8	40.4 ± 2.5			
90 d	39.9 ± 2.9	38.3 ± 2.6			
150 d	41.1 ± 3.0	38.7 ± 2.7			
			0.102	0.006	0.116

Tab.1 Blood parameters in relation to dietary treatment and sampling time

The concentration of serum haptoglobin and AST showed a time effect but no differences in relation to the dietary treatment or to the duration of treatment.

The total antiradical activity of whole blood showed an almost significant effect of treatment ($P = 0.09$) (Figure 1). There were no significant differences at the level of red blood cells.

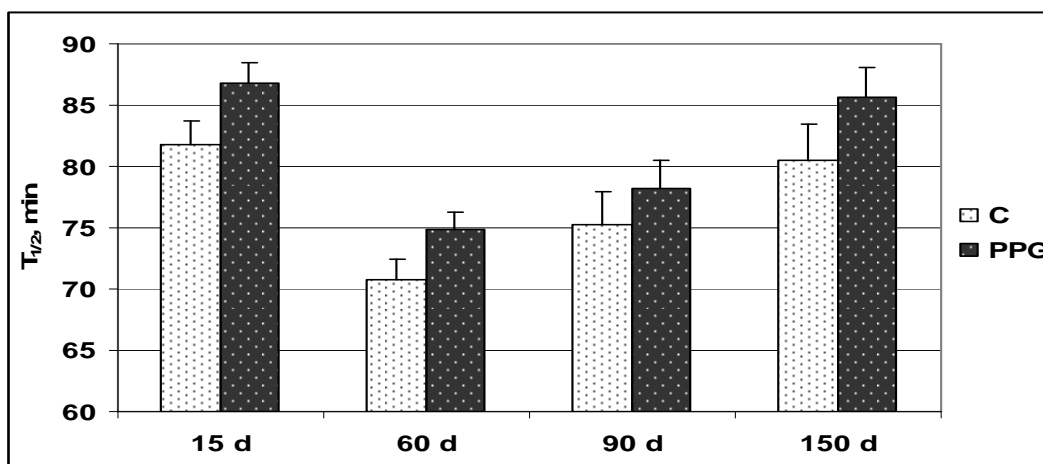


Fig. 1. The total antiradical activity of whole blood of piglets as a function of days post weaning and dietary treatment: control diet (C) or diet supplemented with phenylpropanoids (PPG).

6.5 Conclusions

In literature, the effects on performance due to integration with natural antioxidants are quite contradictory. In this test, our results are in agreement with what has been shown by Neill (2006) who found similar increases and conversion in the control group and treated with natural antioxidants.

The traditional welfare indicators didn't show any effect of treatments and the decreasing trend of haptoglobin is consistent with the literature (Sauerwein et al., 2005), showing a maximum concentration at around 5 weeks of life, corresponding to the removal of the piglet from the mother, and the subsequent reduction in serum in the following weeks. This logic also applies to the AST whose concentration is highest for both treatments at 15 days post weaning, when the inflammatory processes occurring more frequently.

The almost significant treatment effect on total antiradical capacity demonstrates the particular sensitivity of the method for assessing pig welfare than the other blood parameters investigated.

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CHAPTER 7

KRL test to objective evaluation of welfare: assessment of sensibility to housing conditions

7. KRL test to objective evaluation of welfare: assessment of sensibility to housing conditions

7.1 Abstract

The aim of this work was to determine the sensibility of KRL test to evaluate pig welfare in relation to different housing conditions. It is reported that the response to oxidative stress could be considered as welfare parameter in swine. The KRL test allows the evaluation of total blood antioxidant activity. In the trial we evaluated the total blood antioxidant activity of 12 swine allotted respectively to solid and totally slatted floor. The results showed that the total antioxidant activity of red blood cells resulted higher in pig allotted to the solid floor ($P=0.01$). The present research showed that the KRL test is able to discriminate welfare in relation to different housing conditions.

7.2 Introduction

The management of production animals has changed radically across the European Union (EU) over the past five decades. Over this time, animal agriculture has intensified and the animals have been moved to indoor housing systems with higher stocking densities. Over the past years a considerable amount of scientific research has focused on animal welfare (Broom 1991; Sandøe et al., 2003). Performance records, behavioural, physiological and clinical parameters are considered as good indicators for assessing animal welfare (McGlone, 2001; Broom 1996). The welfare in pig species could be evaluated through three different basic approaches. The first method is based on the normal biological functioning including the physical and physiological condition of the animal. The second approach regards animal feelings (Broom 1991) and it can be assessed using ethological parameters. The third, called functional approach, is based on physiological, immunological and pathological indicators, that currently offers interesting data on pig welfare (Barnett et al., 2001).

In the recent years oxidative stress became an important goal in human and animal research as cause of some disease. The “reactive oxygen metabolites” (ROM) are produced endogenously by normal metabolic processes, but amounts may be increased markedly by exogenous factors (Machlin and Bendich, 1987). Deficiencies of natural protective substances or excess exposure to stimulators of ROM production may result in oxidative stress, defined as an imbalance between oxidants and antioxidants at the cellular or individual level (Finkel and Holbrook, 2000).

In farm animals, oxidative stress may be involved in several pathological conditions, that are relevant for animal production and the general welfare of the individuals (Lykkesfeldt et al., 2007). Diseases such as pneumonia and sepsis have been shown to involve altered redox balance in pigs (Lauritzen et al., 2003; Basu and Eriksson, 2000). Brambilla et al., (2002) reported that the response to oxidative stress can be utilized as welfare parameters in pigs.

Methods for quantifying oxidative stress include the assessment of the total antioxidant capacity of serum or plasma. The Trolox equivalent antioxidant capacity (TEAC) assay, the oxygen radical absorbance capacity (ORAC) assay, and the ferric reducing ability of plasma (FRAP) assay are commonly used and have been extensively evaluated. These methods allow to measure the total antioxidant capacity of serum or plasma without considering the antioxidant defences in the red blood cells.

The KRL test shows the resistance to free radicals assessed as the time needed to haemolyse 50% of red blood cells exposed to a controlled free radical attack and provides an assessment of total antioxidant defenses, since all families of antioxidants present in whole blood are used to fight off the oxidant attack (Stocker et al., 2003; Girard et al., 2005). The KRL test has several applications; in humans KRL is used to study the effectiveness of natural or pharmaceutical treatments and to discriminate welfare conditions depending on medium or high stress.

7.3 Materials and methods

This trial was performed to test the KRL test sensibility to discriminate the effect of the housing conditions on total blood antioxidant activity in pigs.

In the experimental trial 24 barrows of a live weight in the range of 80 to 120 kg, half allotted to solid floor and half to totally slatted floor, were randomly selected. Fasting blood samples were taken by anterior vena cava puncture, collected in 10 mL vacutainer glass tubes containing EDTA (Venoject®, Terumo Europe N.V., Leuven, Belgium) and immediately placed on ice pending analysis. The analysis were performed within 24 h from the sampling procedure.

Total antiradical activity of whole blood and red blood cells (RBC) for each pig was evaluated using KRL biological test (Laboratoires Spiral, France). The KRL test is currently used to test the capability of erythrocytes to resist a standardized production of free radicals generated from the thermal decomposition of a 27 mmol/L solution of 2,2'-azobis (2-amidinopropane) hydrochloride at 37 °C (Prost, 1992; Blache and Prost, 1992). Whole Blood and RBC samples diluted to 1/50 were submitted in isotonic saline solution to organic free radicals. Haemolysis was recorded using a 96-well microplate reader by measuring the optical density decay at 450 nm (Figure 1).

Results were expressed as the time required to reach 50% of maximal haemolysis (half-haemolysis time - HT₅₀ - in minutes), which refers to the whole blood resistance to free-radical attack.

One-way ANOVA was used to determine statistically significant differences between the two housing conditions. Data are presented as mean \pm SEM. Differences between means were considered significant at $P < 0.05$

7.4 Results and discussion

No differences in the total blood antioxidant activity were observed in pigs reared on the two different kinds of floor ($P = 0.320$) (Figure 1A). Considering the antioxidant activity of the RBC, an higher value was observed in pigs reared on solid floor than those on totally slatted floor ($P = 0.010$) (Figure 1B).

The RBC value concerns especially the intracellular defence status and it is important for understanding the balance between attack and defence of organism in a medium/long period, considering that RBC mean life in pig is 60-85 days (Pastorelli et al., 2009). This result allows to hypothesize that the environmental stress, due to the totally slatted floor, cause an intracellular imbalance between the oxidative and antioxidant systems that make the cells more susceptible to oxidative damage (Brambilla et al., 2002). The above findings point out that the adaptive response to a long term stressing condition implies an impairment of this reaction. During the state of chronic stress negative effects on health and the development of pathological conditions involving oxidative stress may occur (Lykkesfeldt and Svendsen, 2007).

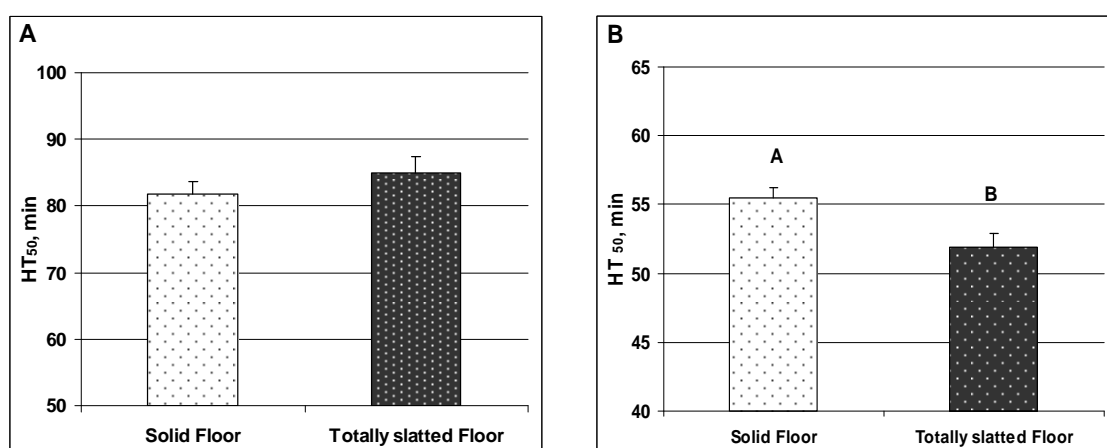


Figure 1. Whole blood (A) and red blood cells (B) antioxidant activity in pigs reared on solid or totally slatted floor¹.

¹Data are reported as means \pm SEM; n=12; A, B for $P = 0.010$.

7.5 Conclusions

Under the conditions of the present research, the KRL test is able to discriminate pig welfare in relation to different housing conditions. The housing system with totally slatted floor negatively influenced RBC antioxidant activity, evidencing a sign of a chronic stress. This result confirm that the KRL test should be consider as a new laboratory analysis to assess welfare in pig species.

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CHAPTER 8

General Discussion

8. General discussion

In literature there are few data on oxidative stress in farm animals, in recent times has increased the interest in this parameter having correlation with the onset of many diseases.

Analysing the results of the experimental trials, it is evident that oxidative stress has an important role at a critical stage of pig farming: the period after weaning. The post-weaning is a very delicate phase in which drastic morphological and physiological changes occur, particularly of the gastrointestinal tract (Pluske et al., 1997; Owusu-Asiedo et al., 2003).

Data obtained with KRL test are consistent with studies reporting a decrease in antioxidants in the post weaning, resulting in increased oxidative stress. Sauerwein et al. (2005) showed that in the post weaning there is an increased production of ROS concomitant with the increase in serum haptoglobin, index of inflammatory processes.

In addition, Lauridsen and Jensen (2005) reported a decrease in the serum level of vitamin E deficiency with onset of symptoms compatible with an increase in oxidative stress. Vitamin E is essential for the development of the immune system and can counteract oxidative stress in piglets after weaning. The diet therefore plays a key role on body function and growth performance of piglets. The KRL data show that proper integration of antioxidants in the post weaning can produce positive effects on the piglet's defenses against free radicals, resulting in improved overall health status and welfare.

In intensive pig farming, the relationship between housing system and welfare have become an important target of interest (EFSA, 2005).

In the experimental trial 5, KRL test was applied to assess welfare, defined as total radical scavenging capacity, in two groups of pigs, one consisting of individuals reared on solid floor, the other of subjects of the same age, genetic type and diet, but raised on totally slatted floor.

No differences in the total blood antioxidant activity were observed between the two groups, while the antioxidant activity of the erythrocytes was higher in pigs reared on solid floor than on pigs reared on totally slatted floor.

This result is very interesting because KRL analysis of RBCs antioxidant defences reflects the free radical aggression of the last two-three months of life of the animal, being the average life span of RBCs 60-85 days, while KRL analysis of whole blood gives an idea of total antioxidant defences of the organism at the time of sampling.

The pigs subjected to chronic stress, as in case of uncomfortable slatted floor, greater use their antioxidant reserves to keep in good condition (Corino et al, 2009)

In a study involving 90 pig herds, Gillman et al. (2009) observed several lesions, mainly bursitis, erosion and detachment of leaflets at the distal ends of the limbs. They demonstrated that the lesions was correlated to the type of floor (solid or slatted) utilized in the herds and showed that floor type can affect the health, performance and behavior of animals.

In addition, it was demonstrated that the type of floor is responsible for some changes in the carcass. In a study of 160 kg pigs, the animals reared on totally slatted floor showed a significantly higher fat thickness compared to those grown on solid floor; pigs allotted to slatted floor reduce movements to avoid injuries (Rossi et al., 2008).

The trial results allow to hypothesize that environmental stress causes an imbalance between intracellular oxidative and antioxidant systems, making the cells more susceptible to oxidative damage.

During the state of chronic stress negative effects on health and the development of pathological conditions involving oxidative stress may occur (Lykkesfeldt and Svendsen, 2007).

As shown by the trials, the KRL test has proven to be a sensitive test to detect a higher total antiradical capacity in all situations connected to higher welfare and to provide additional objective data on the adaptive problems of animals during the post weaning period. It might be useful to further investigate the different radical scavenging activity by assessing the total possible sources of variation and influence of this parameter, such as the different phases of the reproductive cycle of the sow, the genetic type, management, therapeutic interventions, environmental parameters. In the near future is expected to test the application on other matrices (meat, natural extracts, feed, etc ...) in order to gain a more detailed overview of all the variables related to oxidative stress.

CHAPTER 9

References

9. References

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CHAPTER 10

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