Research in Veterinary Science 94 (2013) 372–377

Contents lists available at SciVerse ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

Application of KRL test to assess total antioxidant activity in pigs: Sensitivity to dietary antioxidants

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ARTICLE INFO

Article history:
Received 25 January 2012
Accepted 12 August 2012

Keywords:
Dietary antioxidant
Total antioxidant activity
KRL test
Pig

ABSTRACT

The application of Kit Radicaux Libres (KRL) test to assess total blood antioxidant activity in pigs was evaluated. The KRL has been validated and is widely used in humans for assessing the effectiveness of natural or pharmaceutical treatments, and *in vitro* to evaluate the antioxidant activities of natural or synthetic antioxidants. In this study the sensitivity of the KRL test in assessing the effectiveness of dietary antioxidant supplementation (vitamin E and plant extract) was evaluated in two different phases of pig breeding. The first trial, in post-weaned piglets (40 piglets/group) fed dietary vitamin E supplementation for 60 days, indicated that there was a higher total antioxidant activity ($P = 0.032$) of whole blood and of red blood cells ($P = 0.001$) than for control pigs. The second trial indicated that long-term supplementation of water soluble plant extract (20 pigs/group) from the leaves of Verbenaceae (*Lippia* spp.) tended ($P = 0.091$) to increase antioxidant activity in the whole blood of treated, rather than control pigs. These results indicate that the KRL might be recommended as one of efficient means for evaluating antioxidant activity of dietary ingredients fed to pigs.

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1. Introduction

In recent years increasing experimental data has provided evidence for the involvement of oxidative stress in the development of many diseases. In farm animals, oxidative stress may be involved in several pathological disorders, including those relevant to health and animal production (Lykkesfeldt and Svendsen, 2007).

Reactive oxygen species (ROS) have biological functions essential for normal physiology, and production of ROS may be increased considerably in response to various stimuli (Machlin and Bendich, 1987). These include extracellular factors signaling through plasma membrane receptors, such as hormones, pro-inflammatory cytokines and physical and environmental factors like UV stress, drug and pathogen invasion (Bashan et al., 2009). Deficiencies in antioxidant substances or excess exposure to stimulators of ROS production may result in oxidative stress, defined as an impaired homeostasis between oxidants and antioxidants at a cellular level (Finkel and Holbrook, 2000).

Oxidative stress can be measured directly by detecting free radical production, or indirectly by detecting antioxidant molecules or oxidative damage biomarkers. In general, free radical production can be readily detected, but it is extremely difficult to assessing *in vivo* the total antioxidant activity. Antioxidant may be present within cells, cells membranes and in extracellular fluids. Intracellular antioxidants include glutathione which can quench free radicals and enzymes, such as catalase, glutathione peroxidase, and superoxide dismutase, which can decompose the oxidants. Membrane bound antioxidant include α-tocopherol, β-carotene and ubiquinone (Halliwell and Gutteridge, 1989). The antioxidant system in plasma is mostly accounted for by low molecular weight antioxidants (ascorbic acid, glutathione, tocopherols, phenolic compounds) of a dietary origin (Evans and Halliwell, 2001). The concentration of individual antioxidants is generally not representative of the total antioxidant activity due to the synergistic and antagonistic interactions among antioxidants. Thus, total antioxidant activity may give more biologically relevant information than merely measuring concentrations of individual antioxidants (Somogyi et al., 2007). There are many methods for assessing oxidative stress (Monaghan et al., 2009). Table 1 shows several methods that assess the total antioxidant capacity in plasma/serum. The Kit Radical Libres (KRL) test is a biological test that evaluates the antioxidant status of an organism by testing the antioxidant defence systems of both plasma and red blood cells (RBC) (Prost, 1992). The KRL test evaluates the total antioxidant activity of blood by measuring the time required to haemolyse 50% of the RBC exposed to a controlled free radical attack. The KRL test has been validated for humans and has several *in vivo* and *in vitro* applications. In humans, the test has been used to study the effectiveness of natural or

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http://dx.doi.org/10.1016/j.rvsc.2012.08.005

Table 1
Methods to assess antioxidant capacity.

Method	Sample	Principle	References
OXY-adsorbent test	Plasma	Colorimetric assessment of ability of the anti-oxidant barrier to cope with the oxidant action of hypochlorous acid	Iorio (2004)
Total antioxidant activity (TAS)	Plasma/serum	Colorimetric assessment of sample capacity to quench and decolorize chromogenic-free radical	Rice-Evans and Miller (1994) Re et al. (1999)
Oxygen radical absorbance capacity (ORAC)	Plasma/serum	Colorimetric assessment of sample antioxidant capacity in presence of 2,2′-azobis(2-amidinopropane) dihydrochloride	Cao and Prior (1998)
Trapping antioxidant parameter (TRAP)	Plasma	Measure of oxygen consumption during a controlled lipid peroxidation reaction induced by thermal decomposition of an azo-compound	Wayner et al. (1985)
Ferric reducing antioxidant power (FRAP)	Plasma	Colorimetric assessment of sample antioxidant potential by measuring its ferric reducing ability, in presence of ferric chloride ($FeCl_3$)	Benzie and Strain (1996)
Total oxidant scavenging capacity (TOSC)	Plasma/serum	Gas chromatographic assessment capacity of antioxidants against hydroxyl radicals, peroxyl radicals and peroxynitrite	Winston et al. (1998)
Cupric reducing antioxidant capacity (CUPRAC)	Plasma/serum	Colorimetric assessment of sample antioxidant potential in the presence of bis(neocuproine) copper(II) chelate	Apak et al. (2005)
KRL test	Whole blood/ red blood cell	Time needed to haemolyse 50% of red blood cells when sample is under a free radical attack (AAPH)	Prost (1992)

pharmaceutical treatments (Girodon et al., 1997). In this respect, the KRL test has been used to distinguish between low dose supplementation of different antioxidant vitamins in elderly patients (Lesgards et al., 2002). Moreover the antioxidant capacity of mononuclear cells was also evaluated (Caspar-Bauguil et al., 2009). For *in vitro* studies, the test has been used to assess the antioxidant potential of synthetic and natural substances (Blache et al., 1991; Lesgards et al., 2005; Rossi et al., 2009b).

Some studies on the antioxidant status of animals, using the KRL test, have been published, particularly on rats (Durand and Blache, 1996; Blache et al., 2002; Boukortt et al., 2004; Taleb-Senouci et al., 2009), birds (Alonso-Alvarez et al., 2004; Bertrand et al., 2006), and rabbits (Brzeziska-lebodziska, 2001). However, there have been few studies on intensively reared animals. The objective of this study was to determine the sensitivity of the KRL test in pigs fed dietary supplementation of liposoluble and water soluble antioxidants in different breeding phases.

2. Material and methods

Two independent trials were performed to evaluate the sensitivity of the KRL test for identifying the effects of fat and water soluble antioxidant supplements on total blood antioxidant activity of pigs. In the first trial, dietary supplementation with vitamin E was performed during the post-weaning period. In the second trial, long-term supplementation with plant antioxidants from weaning to slaughter was evaluated. The dosage of plant extract in the feed was chosen on the basis of our previous study in pig (Corino et al., 2007). The dosage of vitamin E in the feed was chosen on the basis of KRL test results that compared the antioxidant activity of Vitamin E and plant extract (Rossi et al., 2009b). All procedures involving animals were carried out in accordance with European Communities Council Directive (86/609/EEC, 1986) and approved by the Italian Ministry of Health (L. n. 116/92).

2.1. Liposoluble antioxidant supplementation

Eighty weaned Dalland piglets (24 days of age), 40 castrated males and 40 females, weighing 7 ± 0.5 kg, were randomly selected and allotted to two dietary treatments: control diet (C, 175 mg/kg of α-tocopheryl acetate) and a diet supplemented with the control level plus 54 mg/kg of α-tocopheryl acetate (Vit E, 225 mg/kg of α-tocopheryl acetate). The experimental groups were balanced for body weight and sex. The animals were individually identified and divided into 10 pens (8 piglets/pen) and reared in an environmentally-controlled room. The experimental diets were formulated to meet or a little exceed the requirements for all nutrients (NRC, 1998), and were presented for *ad libitum* consumption. Weight and feed consumption were recorded and the average daily gain (ADG) and feed conversion ratio (FCR) were calculated. On 10 randomly selected castrated male piglets per treatment (two piglets/pen), fasting blood samples were obtained at weaning, 15 and 60 days post-weaning by anterior vena cava puncture. The blood samples were collected in 10 mL vacutainer glass tubes containing EDTA (Venoject®, Terumo Europe N.V., Leuven, Belgium), and immediately stored at 4 °C. Analyses were performed within 24 h of collection.

2.2. Water soluble antioxidant supplementation

In the second trial, the effect of plant antioxidant supplementation was evaluated in castrated male pigs from weaning to 99.7 ± 0.5 kg LW on total blood antioxidant activity. Forty weaned Dalland piglets (24 days of age), 20 castrated males and 20 females, weighing 7 ± 0.4 kg, were randomly selected and allotted to two dietary treatments: control diet (C) and an experimental diet supplemented with 1 kg/t of plant extract (PE), titrated in phenyl propanoid glycosides expressed as verbascoside. The antioxidant supplement contained a water-soluble extract of Verbenaceae (*Lippia* spp.) leaves, prepared on an industrial scale by a standardised procedure that included ultrasonic extraction with 60% aqueous ethyl alcohol followed by spray-drying with maltodextrins as an excipient. To avoid oxidation in the complete feed, the supplement was microencapsulated within a protective matrix of hydrogenated vegetable lipids using spray cooling technology (Sintal Zootecnica, Isola Vicentina, Vicenza, Italy).

The experimental groups were balanced for body weight and sex. The animals were individually identified and divided into 10 pens (four pigs/pen) and reared in an environmentally-controlled room. The experimental diets were formulated to meet or a little exceed the requirements for all nutrients (NRC, 1998), and were presented for *ad libitum* consumption. Piglets weight and feed consumption were recorded and the average daily gain (ADG) and feed conversion ratio (FCR) were calculated. On 10 randomly selected castrated male pig per treatment (2 pigs/pen), fasting blood sam-

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