Assessment of a natural dietary extract, titrated in phenylpropanoid glycosides, on blood parameters and plasma oxidative status in intensively reared Italian hares (Lepus corsicanus)

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Two different doses of a natural extract titrated in phenylpropanoid glycosides (PPGs) were evaluated for their effect on blood parameters and plasma oxidative status in pairs of intensively reared Italian hares. The study lasted 210 days, during which 45 couples of hares were divided into three homogeneous groups. A control group (CON) was fed a control diet while the two experimental groups were fed a diet supplemented with 1 or 2 kg/t of a supplement titrated in PPGs. Blood samples were obtained at 0, 70, 140 and 210 days and assayed for plasma lipid profiles, bilirubin, haematological parameters and indicators of oxidative status (reactive oxygen metabolites (ROMs), thiobarbituric acid reactive substances (TBARS), vitamins A and E). Although dietary treatment did affect the levels of triglycerides, total cholesterol and total bilirubin, all of which decreased markedly ($P < 0.05$), while significantly increasing the ($P < 0.01$) HDL cholesterol values, it also significantly improved the oxidative status of the blood, which displayed an increase in both vitamin E ($P < 0.01$) and vitamin A ($P < 0.05$) and a decrease in ROMs ($P < 0.01$) and TBARS ($P < 0.05$). The improvements in the blood parameters, lipid profile and plasma oxidative status continued to increase significantly as the trial progressed, indicating a positive effect with increased length of treatment. The results of this study demonstrate an important role for feed supplementation with respect to antioxidant activity on some blood parameters, including the lipid profile and the oxidative status of blood.

Keywords: natural extracts, verbascoside, blood parameters, hare

Implication

The use of natural extracts, titrated in phenylpropanoid glycosides (PPGs), may improve blood parameters and promote the welfare of intensively farmed hares. This can positively affect the health status and resistance to adverse conditions in breeding couples, allowing them to reproduce and be relocated to areas for restocking. The utilisation of an antioxidant supplement, titrated in PPGs, can provide greater resistance to parasites and disease upon release in a restocking area. It is thus possible to counteract the decline of native species and maintain biodiversity.

Introduction

Free radicals are highly reactive molecules produced under normal biologic conditions, mainly during oxygen consumption in redox reactions that are required to generate energy and eliminate xenobiotic and pathogenic organisms. An excess of free radicals can markedly impair cellular metabolism and significantly damage tissues. Organisms have natural protection mechanisms in the form of enzymatic and chemical detoxification systems against excessive free-radical attack. Thus, under normal physiologic conditions, a balanced state is established between free-radical production and anti-radical defences.

Nevertheless, various lifestyle, nutritional and environmental defences alter the balanced state and result in so-called oxidant stress. This impairment of the individual’s overall defence capacity can then favour the development of many diseases.

Many substances naturally present in plants, such as phenolics and flavonoids, have the ability to neutralise free radicals. Some of these substances inhibit chain reactions that lead to the formation of additional radicals, thus preventing the propagation of cellular damage. Other substances
Material and methods

Animals and diets

The study lasted 210 days (February to August) and was conducted at the farm ‘Allevamenti Roger’ in the countryside of Isernia, Molise Region, with 45 pairs of Italian hares that were intensively reared in French model cages (Castiglione et al., 1996). The animals were uniform in live weight (males: 3.34 and 3.81 kg females), age (2 years ± 0.6) and order (all females were multiparous). The hares were divided into three groups of 15 pairs each: a control group (CON) and two experimental groups (T1 and T2). The experimental groups were fed a diet supplemented with 1 or 2 kg/t of a commercial antioxidant, which was produced and provided by Consorzio Powerfeed (Costa de’ Nobili, 27010 Pavia, Italy). The antioxidant supplement contains a water-soluble extract of Verbenaceae (Lippia spp.) leaves, prepared on an industrial scale by a standardised procedure that includes ultrasonic extraction with 60% aqueous ethyl alcohol (EtOH) followed by spray drying with maltodextrins as an excipient.

The PPGs and benzoic acid content of the feed supplement are reported in Table 1, according to a certificate of analysis provided by the manufacturer. The quantitative analysis of the phenolic compounds was performed by HPLC-UV-DAD (Rastrelli L, personal communication) according to Piccinelli et al. (2004). To avoid oxidation in the complete feed, the supplement is microencapsulated within a protective matrix of hydrogenated vegetable lipids using spray-cooling technology (Sintal Zootecnica, Isola Vicentina, Vicenza, Italy).

A common feeding programme was used for all hares and consisted of a diet (Martini Spa, Budrio di Longiano FC, Italy) offered ad libitum as 4-mm pellets. The ingredient composition and the determined (AOAC, 2000) nutrient value of the diet are shown in Table 2.

The hares also had ad libitum access to alfalfa hay. Breeding couples were observed during 7 months for several reproductive cycles and subjected to the following experimental measurements:

1. BW of males and females at 0 day (trial start), 70, 140 and 210 days.
2. Weekly feed intake, collected for breeding pairs and grouped in periods 0 to 70, 70 to 140 and 140 to 210 days.
3. Blood samples at 0, 70, 140 and 210 days.

Blood samples were collected in the morning from the external ear vein by immobilising the animal in a tissue bag, from which only the ears protruded through the slots. The bag, made to fit the animal, maintained their stillness with darkness to keep them calm. Blood samples were collected in two 2-ml vacutainer glass tubes (Venoject, Terumo Europe N.V., Leuven, Belgium): the first contained sodium heparin and the second contained EDTA for measuring haematological parameters. The sodium heparin blood samples were centrifuged for 15 min at 3000 r.p.m.

Blood analyses

Triglycerides, total cholesterol, HDL cholesterol and bilirubin levels in plasma were immediately determined with an automated clinical chemistry analyzer, model ARCO (Biotechnica Instruments S.p.A., Roma, Italy).

The concentration of reactive oxygen metabolites (ROMs) in plasma was determined by a spectrophotometer and a colorimetric method, as proposed by Diacron, using a specific commercial kit at a wavelength of 505 nm (Cesarone et al., 1999). The results were expressed in Carr Units (1 Carr Unit equals 0.024 mmol/l of H2O2).

Table 1 PPGs and benzoic acid content of feed supplement

<table>
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<tr>
<th>Items</th>
<th>g/kg</th>
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<tr>
<td>Gallic acid</td>
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<tr>
<td>3,4-dihydroxybenzoic acid</td>
<td>0.45 ± 0.04</td>
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<td>Methyl gallate</td>
<td>1.915 ± 0.09</td>
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<tr>
<td>Isoverbascoside</td>
<td>0.435 ± 0.04</td>
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<td>Verbascoside</td>
<td>4.470 ± 0.08</td>
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PPGs = phenylpropanoid glycosides.
The determination of thiobarbituric acid reactive substances (TBARS) was performed in plasma according to Esterbauer and Zollner (1989). Briefly, a standard curve was generated using 1,1,3,3-tetramethoxypropane (Sigma Aldrich, St. Louis, USA). Trichloroacetic acid 10% (v/v) was added to the plasma samples, promoting the precipitation of proteins. The resulting mixture was incubated for 15 min on ice. After centrifugation at 2200 r.p.m. at 4°C for 15 min, 0.67% thiobarbituric acid was added to the supernatant. The mixture was incubated in a water bath at 90°C for 10 min, after which the absorbance was read at 532 nm in a spectrophotometer. The results were expressed as μmol of thiobarbituric acid per litre of plasma.

Vitamins A and E were extracted from plasma samples with chloroform (Zhao et al., 2004) and analysed on an HPLC system (Kontron Instruments, Milano, Italy) consisting of an autosampler (HPLC Autosampler 360, Kontron Instruments, Milano, Italy) with a 20 μl loop, a high pressure mixing pump and a 5 μm, 250 × 4.60 mm C18 column (Phenomenex, Torrance, CA, USA). The mobile phase was 100% methanol at a flow rate of 1.0 ml/min. A fluorimeter detector (SFM) and computer with Kroma System 2000 software were used. The concentrations of vitamins A and E were determined by using an internal standard and the elution time of pure standards.

Whole blood was tested for haematological parameters, including red blood cells, white blood cells, platelets and haematocrit, using the automatic blood cell counter SEAC (Radim Company, Calenzano, FI, Italy).

Statistical analysis
After assessing whether the frequency distribution assumed normality, all variables were subjected to a variance test using the GLM procedure for repeated measures in the SPSS program (2009). The fixed effects of dietary treatment and time, as well as their interaction, were included in the model. No significant differences in blood parameters or feed intake between males and females were found, and therefore the experimental unit was a couple of hares. Differences were considered significant at P < 0.05.

Results
Live weight and feed intake
The live weight and feed intake of breeding pairs did not show any significant changes as a result of the experimental treatment.

Blood parameters
The dietary treatment significantly affected (P < 0.05) all parameters listed in Table 3 (triglycerides, total cholesterol, HDL cholesterol and bilirubin).

Triglyceride values were significantly lower for the T1 and T2 groups than for the control group CON at both 140 days (−4.3% and −4.1%, respectively) and 210 days (−6.9% and −8.5%, respectively). The length of the dietary treatment resulted in a marked decrease in triglycerides (P < 0.01), to 7.4% and 8.2% in the T1 and T2 groups, respectively, from the beginning to the end of the trial. The control group did not display significant variation over the same period.

Dietary treatment also produced a decrease (P < 0.05) in total cholesterol by 140 days that continued until 210 days (−4.3% and −4.1%, respectively) and 210 days (−6.9% and −8.5%, respectively). The effect of time was observed as follows (P < 0.01): total cholesterol values decreased in the T1 and T2 groups from the first to the fourth sampling by 9.1% and 8.9%, respectively. The control group, however, did not present appreciable changes during the same period.

Table 2 Ingredients and chemical composition of diet (% as-fed basis unless otherwise indicated)

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DM = dry matter.

aSupplied per kg of diet: 7,000 I.U. vitamin A (trans-retinyl acetate); 1,600 I.U. vitamin D3 (cholecalciferol); 20 I.U. vitamin E (all-rac-tocopherol-acetate); 1.0 mg vitamin K3 (bisulphate menadione complex); 0.7 mg thiamin (thiamine-mononitrate); 3.0 mg riboflavin; 9 mg pantothenic acid (D-Ca pantothenate); 15 mg vitamin B2 (niacin); 100 mg choline (colline chloride); 1 mg pyridoxine (pyridoxine HCl); 0.016 mg vitamin B12 (cobalamin); 16.5 mg Cu (CuSO4 7H2O); 75 mg Fe (FeSO4 7H2O); 40 mg Mn (MnO2); 110 mg Zn (ZnO); 0.1 mg Co (CoSO4 7H2O); 0.2 mg Se (Na2SeO3); 0.8 mg I (Ca(IO3)2) and 125 mg ethiochin.

b Respectively 0, 0.1 and 0.2 kg for Control (CON), Low (T1) and High (T2) level of feed supplement inclusion groups.

n = 2.
At the end of the study (on day 210), HDL cholesterol was higher ($P < 0.01$) in the T1 and T2 groups (20.1% and 22.3%, respectively) compared to the control group. The HDL cholesterol values in the experimental groups also increased significantly by the second (70 days) and third sampling points (140 days). A significant increase ($P < 0.01$) in HDL cholesterol for groups T1 and T2 of 20.1% and 22.3%, respectively, was observed from the beginning to the end of the trial. The control group showed no change over time.

Serum bilirubin decreased ($P < 0.05$) in response to the dietary treatment. At the end of the trial, groups T1 and T2 had lower values of 15.9% and 19.0%, respectively, compared to the control group. Serum bilirubin decreased by 11.7% and 15.5%, respectively, in groups T1 and T2 from the beginning to the end of the trial.

Haematological parameters (RBC, WBC, HGB, HCT, MCV, MCH, MCHC and PLT) did not present statistically significant differences as a result of the dietary treatment over time (Table 4). They were summarised into single data points, such that each parameter was reported as the mean value obtained from the samples taken at 0, 70, 140 and 210 days.

The dietary treatment resulted in a significant improvement in the levels of the markers for oxidative status (ROMs, TBARS, vitamin E and vitamin A; Table 5).

The values of ROMs decreased ($P < 0.01$) from the third sampling time until the end of the trial (210 days) in both the T1 and T2 groups, in which they were lower by 48.1% and 50.6%, respectively, than the control group values. Time had a significant effect on ROMs, such that the values decreased markedly ($P < 0.01$) by 37.6% and 36.3% in groups T1 and T2, respectively, as the trial continued. For the same time interval, the control group CON presented values that increased by 29.2% ($P < 0.05$). TBARS values were also statistically decreased in the T1 and T2 groups, by 70.8% and 73.4%,
respectively \((P < 0.05)\), at the end of the trial (210 days) compared to the control group. The differences with respect to the control group were identified as early as the second sampling time and were maintained until the end of the trial. The length of treatment markedly influenced \((P < 0.01)\) the reduction of TBARS values in groups T1 and T2, which decrease by 39.2% and 40.8%, respectively, from the beginning to the end of the trial. TBARS values in the control group increased by 108.1% \((P = 0.05)\) for the same period.

The blood concentration of vitamin E at the end of the trial was significantly increased \((P < 0.01)\) for groups T1 and T2, by 34.0% and 39.2%, respectively, compared to the control group. In particular, the differences in vitamin E content had emerged by 70 days after the start of the trial in the T2 group, and by 140 days in the T1 group. In addition, an effect for time on vitamin E content was observed \((P < 0.01)\). The content increased by 32.9% and 37.7% in the T1 and T2 groups, respectively, whereas vitamin E content in the control group remained unchanged over the same period.

The content of vitamin A increased significantly during the trial \((P < 0.05)\), by 41.8% and 49.6%, respectively, in the T1 and T2 groups compared to the control group CON. Increases were recorded for the treated groups, starting from the third sampling time point. Throughout the experiment, groups T1 and T2 showed significant increases \((P < 0.05)\) of 37.7% and 49.6%, respectively, in vitamin A concentration, while the control group values remained constant.

### Discussion

Dietary treatment with an antioxidant supplement titrated in PPGs did not influence the BW or feed intake of pairs of hares. The values remained normal for the species throughout the trial \((P = 0.05)\).

The values of blood parameters all conformed to the normal ranges for this species \((P = 0.05)\), although some were influenced by the treatment, which resulted in improved lipid blood values in hares from groups T1 and T2. In particular, significant reductions in triglycerides and total cholesterol and a marked increase in HDL cholesterol were observed. The increase in plasma concentrations of HDL cholesterol might have been caused by the effect of polyphenols that are involved in the regulation of lipid and glucose metabolism.
mechanism of PPARα activation by polyphenols, leading to an induction of lipoprotein lipase (LPL) in peripheral tissues and increased lipolysis, which would result in reduced levels of circulating triglycerides and very low-density lipoproteins (VLDL).

Radwan et al. (2008) fed hens diets supplemented with vitamin E, thyme oil, oregano, rosemary and turmeric, and observed a significant decrease in total lipids for the groups receiving oil of thyme and rosemary. No effect was observed for levels of total cholesterol and LDL cholesterol. In broilers fed diets with thyme leaves, Radwan (2003) and Case et al. (1995) observed a reduction in total lipids and total cholesterol, which was probably due to an inhibiting effect of thymol and carvacrol on HMG-CoA reductase, the enzyme responsible for cholesterol synthesis in the liver. Ali et al. (2007) noted a marked decrease in total cholesterol, LDL cholesterol, triglycerides and total lipids in hens that received feed supplemented with thyme plants.

The decrease in bilirubin levels that we observed is most likely due to the antioxidant activity of polyphenols that inhibits the biochemical pathway involved in bilirubin synthesis (Aliyu et al., 2007). Similarly, Youssef et al. (2003) observed that rabbits fed a diet enriched with antioxidants, such as ascorbic acid, had lower bilirubin levels after daily ingestion of aflatoxin B1.

The experimental treatment described here led to an improvement in the oxidative status of blood plasma, and these results are probably due to the action of antioxidant polyphenols in verbascoside. As redox-active molecules, polyphenols can be oxidised and reduced without them becoming highly reactive free radicals. They play a protective role against ROS, thus reducing the liperoxidases evidenced by the decrease in plasma MDA. The reduction in ROMs and lipid peroxidation (TBARS) can be due both to the direct action of the antioxidant verbascoside, which captures free radicals during the propagation of a chain reaction, and a phase-lock initiation of oxidation by inhibiting pro-oxidant enzymes (Miller et al., 1993; Kamiloglu et al., 2006). These results are in agreement with those reported by Corino et al. (2007b) on weaned piglets fed a verbascoside-supplemented diet. Susca et al. (2005) also observed that Charolais cattle subjected to transport stress had a decrease in the concentration of Malonaldehyde (MDA) when their diet was supplemented with high antioxidant plant extracts. Li et al. (1999) observed a decrease in the concentration of ROMs in a study on skeletal muscle in rats that had been subjected to stress, but whose diet had been supplemented with verbascoside. Similar results were also reported by Fidan and Dundar (2008), who showed that the administration of Yucca Schidigera and Quillaja Saponaria extracts, both rich in saponins, resulted in decreased MDA in diabetic rats, due to the protective antioxidant effects of phenolic groups, such as resveratrol, present in these plant extracts. The increase in serum levels of vitamins A and E can be attributed to the ability of verbascoside to strengthen and save the endogenous antioxidant system, controlling oxidative metabolism by reducing the production of reactive radical species and increasing the antioxidant activity of enzymes (Princen et al., 1998; Zhu et al., 1999; Liao and Yin, 2000).

Moreover, results similar to those presented here were obtained in a study on lambs supplemented with verbascoside (Casamassima et al., 2009). In addition to being stimulated by antioxidants, the increase in plasma values of vitamins A and E is also influenced by the integration of these vitamins in the diet (Mahan, 1991; Hidirogolu et al., 1993; Njeru et al., 1994; Corino et al., 1999; Oriani et al., 2001; Lauridsen et al., 2002).

Conclusion

The experimental treatment did lead to a significant reduction in triglycerides, total cholesterol and bilirubin, as well as a marked increase in serum levels of HDL cholesterol.

The verbascoside also resulted in improved homoeostatic plasma stability, due to a significant decrease in concentrations of ROMs and TBARS and increased concentrations of vitamins A and E.

The results of this research demonstrate an important role for food supplements and antioxidants on some blood parameters, including lipid profiles and oxidative status of plasma, as shown by a marked decrease in ROMs and TBARS and a significant increase in bodily reserves of the antioxidant vitamins A and E.

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