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EFFECT OF LONG TERM DIETARY SUPPLEMENTATION OF *LIPPIA CITRIODORA* EXTRACT ON SEMEN QUALITY TRAITS IN BROWN HARE (*LEPUS EUROPAEUS*)

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ABSTRACT

There is an internationally growing interest concerning application of natural extract sources in animal production area in order to improve the husbandry welfare and the performance. The aim of the present study was to evaluate the effect of dietary supplementation with a natural extract of *Lippia citriodora*, containing verbascoside as main component, on some quality traits of semen of hare, monitoring also the welfare status of animals.
Hares were randomly divided into four groups of 3 animals each, homogeneous by age and body weight, and fed *ad libitum* and free access to water until the end of the trial. Animals were fed for 240 days a commercial diet assigned to four dietary treatments: control diet (CON) and the diet supplemented with 1 g.kg^{-1} of natural extract (low natural extract – LNE) or 1.5 g kg^{-1} of natural extract (medium natural extract – MNE) or 2 g.kg^{-1} of natural extract (high natural extract – HNE). All hares were subjected to the following experimental measurements: weekly relief of feed intake, body weight and blood samples at 0 and at 240 day of trial, and semen collection at 180, 210 and 240 days of trial.
The body weight of the hares and their feed intake were not affected by the experimental treatment. At the end of the trial, sperm volume, pH and sperm concentration values were not effected by *Lippia citriodora* extract treatment, and the mean values recorded were 0.543 ml, 7.4 and 263.25 10^6 per ejaculate, respectively. The dietary treatment negatively affected ($P < 0.05$) the sperm motility values in LNE, MNE and HNE groups.
In conclusion, the results of the present work underline a possible negative effect of the *Lippia citriodora* extract on the semen quality characteristics, besides the improvement in welfare status of the treated hares, reflected in a better lipid profile and an improved plasma oxidative markers.

Key words: antioxidant supplement; biochemical parameters; hare spermatozoa

INTRODUCTION

There is an internationally growing interest concerning application of natural extract sources in animal production area in order to improve the husbandry welfare and the performance. Natural antioxidants have been widely reported to have potent antioxidant, anti-inflammatory and antimicrobic activities related especially to their phenolic content (Pereira *et al*., 2009).

Lippia citriodora, a plant species in the Verbenaceae family, is characterised by the presence of several phenolic compounds, including flavonoids, phenolic acids, luteolin derivatives and phenylpropanoids (Valentão *et al*., 2002) that are the most abundant compounds in Verbenaceae extracts (Pascual *et al*., 2001). Phenylpropanoid glycosides are powerful antioxidants acting by direct scavenging of reactive oxygen and nitrogen species, or as chain-breaking peroxyl radical

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scavengers (Afanasev, 2005).

Previous studies (Palazzo *et al.*, 2011; Casamassima *et al*., 2012, 2015) showed that dietary supplementation with *Lippia* extracts in different species such as hare, sheep and rabbit showed an improvement in biochemical parameters, characterised by a decrease in the concentration of triglycerides, total cholesterol, low density lipoprotein (LDL) cholesterol, bilirubin, reactive oxygen metabolites (ROM) and thiobarbituric acid reactive substances (TBARS), by an increase in high density lipoprotein (HDL) cholesterol and plasma vitamin A and E.

Therefore, the aim of the present study was to evaluate the effect of dietary supplementation with a natural extract of *Lippia citriodora*, titrated in verbascoside, on some quality traits of hare semen, monitoring also the welfare status of animals.

MATERIAL AND METHODS

The experiment lasted 240 days and was performed on the farm "Allevamenti Roger" in the countryside of Isernia (Molise region, Italy) on 12 fertile and healthy hare males (*Lepus europaeus* Pallas, 1778) housed individually. All the breeding procedures and management of animals were conducted in accordance with the European Directive 2010/65/ EU regarding the protection of animals used for scientific purposes.

To ensure that animals adapt to the experimental condition, an adaptation period of 30 days was kept; after that hares were randomly divided into four groups of 3 animals each, homogeneous by age (270 ± 5 days of age) and body weight (2.8 ± 0.2 kg), and fed *ad libitum* with free access to water until the end of the trial (510 day of age). Animals were fed a commercial diet assigned to four dietary treatments: control diet (CON) and diet supplemented with 1 $g.kg^{-1}$ of natural extract (low natural extract - LNE) or 1.5 $g.kg^{-1}$ of natural extract (medium natural extract - MNE) or 2 $g.kg^{-1}$ of natural extract (high natural extract - HNE). The chemical composition of the feed (AOAC, 2000) was following (per kg of dry matter): crude protein 154 g; crude fat 33 g; crude fiber 195 g; Neutral Detergent Fiber 385 g; Acid Detergent Fiber 240 g; ashes 85 g, moisture 111 g. The experimental diets were prepared by adding the natural extract to the basal commercial mashed diet (4 mm pellets). Hares also had *ad libitum* access to alfalfa hay.

The antioxidant supplement contains a water-soluble extract of *Lippia citriodora* leaves (Verbenaceae, *Lippia* NE), prepared on an industrial basis by a standardised procedure that includes ultrasonic extraction with 60 % aqueous ethyl alcohol followed by spray drying with maltodextrins as an excipient. The bioactive components of the feed supplement, according to a certificate of analysis provided by the manufacturer were: verbascoside 4.47 ± 0.08, methyl gallate 1.91 ± 0.09, gallic acid 1.75 ± 0.07, 3.4-dihydroxybenzoic acid 0.45 ± 0.04 and isoverbascoside 0.43 ± 0.04 $g.kg^{-1}$.

The quantitative analysis of the phenolic compounds was performed by HPLC-UV-DAD according to Piccinelli *et al.* (2004). To avoid oxidation in the complete feed, the supplement is micro-encapsulated within a protective matrix of hydrogenated vegetable lipids using spray-cooling technology (Sintal Zootecnica, Isola Vicentina, Vicenza, Italy).

All hares were subjected to the following experimental measurements: weekly relief of feed intake, body weight and blood samples at 0 and 240 days of trial, and semen collection at 180, 210 and 240 days of trial.

Blood samples were collected in the morning from the external ear vein by immobilizing 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. Triglycerides, total cholesterol, HDL cholesterol, LDL cholesterol, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and bilirubin levels in plasma were immediately determined with an automated clinical chemistry analyzer, model ARCO (Biotecnica Instruments S.p.A., Roma, Italy). The concentration of 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 $mmol.l^{-1}$ of H_2O_2). The determination of TBARS was performed in plasma according to Esterbauer and Zollner (1989). The results were expressed as mmol of thiobarbituric acid per litre of plasma. Vitamins A and E were extracted from plasma samples with chloroform (Zhao *et al*., 2004) and analyzed on an HPLC system (Kontron Instruments, Milano, Italy).

Semen samples were collected with electroejaculation method according to Kozdrowski and Dubiel (2005), giving intramuscularly medetomidine (Domitor®) at dose of 0.1 $ml.kg^{-1}$ body weight. After few minutes from achieving full anesthesia, 4 ml of warm physiological saline were infused to the rectum and rectal probe was inserted into rectum as deep as was possible. Semen collection followed the commonly accepted rules, and after four to five impulses the ejaculated liquid was sampled. There were no adverse effects after electroejaculation and the interval between consecutive semen collections was four weeks. Following semen collection the volume of ejaculate was recorded and the percentage of motile spermatozoa was assessed

in light microscope (Alphaphot-2 YS2, Nikon, Tokyo, Japan) equipped with a thermostable table of 37 °C, under 200 x magnification. The concentration of spermatozoa in a whole ejaculate was assessed using Spermacue® photometer (Minitube®, Bio-One GmbH, Germany) and the pH value of spermatic liquid was assessed with a Hanna Instrument pH-meter (Woonsocket, USA) with a specific probe. For all experimental determinations each sample was analyzed in triplicates.

Statistical analysis

After verifying the normality of the frequency distribution, all variables were subjected to a variance test using the GLM procedure for repeated measures in the SPSS program (2010). The fixed effects of dietary treatment and time, as well as their interaction, were included in the model. Differences were considered as significant at $P < 0.05$.

RESULTS AND DISCUSSION

During the whole test period, the welfare status of animal was always considered to be good. The present study was not focused on growth performance of hares since a low number of replicates were used.

The body weight and feed intake of the hares were not affected by the experimental treatment. In general, any effect from the addition of natural extract should not be expected, when animals are healthy and housed in a clean environment. Moreover, the animals used in this experiment were adult.

The dietary treatment with *Lippia citriodora* extract affected ($P < 0.05$) all tested serum biochemical parameters, excepting AST and ALT values (Table 1).

In the three experimental groups, the triglycerides ($P < 0.01$) at the end of the trial were lower than in the CON group. The time affected the triglyceride values; in fact, from the beginning to the end of the test they decreased significantly in the LNE, MNE and HNE groups, whereas in the same period CON-group values were unchanged.

Total cholesterol was influenced by the dietary treatment at the end of the trial, showing a significant decrease in the MNE and HNE groups compared with the CON group. Time effect on total cholesterol values showed a decrease in the MNE and HNE groups, whereas in the CON and LNE group total cholesterol values were unchanged.

The HDL cholesterol increased significantly at 240 d of sampling in LNE, MNE and HNE groups compared to the CON group. A time effect was also observed, from the beginning to the end of the trial. Serum HDL cholesterol was statistically higher in the LNE, MNE and HNE groups, whereas values remained almost unchanged in the CON group.

The LDL cholesterol values were significantly lower in the LNE and HNE groups compared with the CON group at the end of the trial. A time effect was observed during the whole trial on that parameter, decreasing in the LNE, MNE and HNE groups.

The better lipid profile, recorded in the present experimental trial, with the use of *Lippia* NE, through the increase in plasma concentration of HDL cholesterol, may be due to the effect of polyphenols, which are involved in the regulation of lipid and glucose metabolism. According to some authors (Norata *et al*., 2003; Bursill and Roach, 2007), this activates the PPAR-α receptor, with an increased stimulation effect in the liver of the expression of key proteins involved in the metabolism of HDL. Triglycerides would also seem to be involved in the same mechanism of activation of PPAR-α by the polyphenols, with an induction in lipoprotein lipase expression in peripheral tissues and increased lipolysis, which probably results in a reduction in circulating triglycerides and very low density lipoprotein.

Our previous experiments in sheep, hare and rabbit, fed with a dietary NE supplement, revealed a significant reduction in triglycerides, total cholesterol and LDL cholesterol along with increased HDL cholesterol (Palazzo *et al*. 2011; Casamassima *et al*. 2012, 2015). In broilers fed the diet enriched with thyme leaves, Radwan (2003) and Case *et al*. (1995) found a reduction in total lipids and total cholesterol. This was probably due to an inhibiting effect of the enzyme HMG-CoA reductase by thymol and carvacrol, which are responsible for the cholesterol synthesis in the liver.

Serum AST and ALT showed a tendency to decrease in all three experimental groups, with no statistical significance, compared to the CON group. Moreover, values of AST and ALT remained within the normality range of the species, and no hepato-toxicity to animals was found.

Serum values of bilirubin decreased ($P < 0.05$) in the LNE, MNE and HNE groups at the end of the trial due to the dietary treatment. The decrease in bilirubin could be attributed to the antioxidant activity of polyphenols, which inhibits the biochemical mechanisms involved in the formation of the same bilirubin (Aliyu *et al*., 2007).

Table 2 reported data on the plasma oxidative markers in hares fed *Lippia* NE extract.

The ROM values markedly decreased in the LNE, MNE and HNE groups compared to the CON. The duration of treatment resulted in a significant decrease in ROM values in all three experimental groups, whereas in the CON group the concentration during the same period of time remained unchanged.

The TBARS markedly decreased in the treated groups compared with the CON, with a decrease in LNE,

Table 1: Biochemical parameters in hares fed *Lippia citriodora* extract

	Experimental groups§					P-value‡		
Parameters	CON	LNE	MNE	HNE	SEM¶	D	T	D×T
Animals (n)	3	3	3	3				
Triglycerides (mg.dl^{-1})								
0 d	120.7	118.7^{a}	121.7^{a}	118.3^{a}	3.13			
240 d	119.0^{1}	111.0^{2b}	112.0^{2b}	102.2^{3c}	7.18	0.008	0.009	0.049
Total cholesterol (mg.dl^{-1})								
0 d	25.6	23.8	26.0^{a}	24.2^{a}	2.41			
240 d	25.9^{1}	22.4^{12}	19.7^{2b}	20.7^{2b}	3.67	0.225	0.039	0.049
HDL cholesterol (mg.dl^{-1})								
0 d	5.1	5.2^{a}	5.6^{a}	5.5^{a}	1.54			
240 d	4.9^{1}	6.1^{2b}	7.1^{3b}	6.9^{3b}	1.20	0.049	0.048	0.033
LDL cholesterol (mg.dl^{-1})								
0 d	8.1	8.2^{a}	8.2^{a}	8.1^{a}	0.66			
240 d	8.2^{1}	7.1^{2b}	7.2^{2b}	6.6^{2b}	1.05	0.045	0.045	0.038
AST (UI)								
0 d	108.0	107.8	107.7	107.6	2.93			
240 d	111.1	107.5	107.4	107.3	3.84	0.348	0.489	0.445
ALT (UI)								
0 d	56.4	55.2	57.7	58.0	3.19			
240 d	57.3	52.9	54.3	54.9	3.54	0.053	0.125	0.231
Bilirubin (mg.dl^{-1})								
0 d	0.60	0.62^{a}	0.63^{a}	0.60^{a}	0.07			
240 d	0.61^{1}	0.53^{2b}	0.53^{2b}	0.47^{3b}	0.08	0.042	0.015	0.048

§ Control (CON); 1 g.kg^{-1} low natural extract (LNE); 1.5 g.kg^{-1} medium natural extract (MNE); 2 g.kg^{-1} high natural extract (HNE).
¶ SEM: Standard error of means
‡ D: fixed effect of dietary supplementation; T: fixed effect of time; D×T: interaction dietary supplementation x time.
^{1,2,3} Within a row, means without a common superscript differ ($P < 0.05$).
^{a,b} Within a column, means without a common superscript differ ($P < 0.05$).

MNE and HNE groups. The duration of treatment also resulted in a significant decrease in the LNE, MNE and HNE group values, whereas the CON group showed a tendency to increase of TBARS. The experimental treatment led to an improvement in the markers of plasma oxidative status. As a redox-active molecules (capable of being oxidised and reduced without becoming a highly reactive-radical molecule), the NE protects against ROS, with a consequent reduction in lipid peroxidation, as also highlighted by the decrease in plasma levels of TBARS. The reduction in ROM and lipid peroxidation could be attributed both to the direct capture of free radicals due to the antioxidant activity of NE during the propagation phase of the chain reaction, and to a block of the initial oxidative process, through the inhibition of the pro-oxidant enzymes that produce free radicals (Kamiloglu *et al.*, 2006). In our previous research (Palazzo *et al*., 2011; Casamassima *et al*., 2012), we also found an improvement in the markers of plasma oxidative status, in sheep and hare fed *Lippia* NE supplemented in the diet.

In the HNE, MNE and LNE groups, a marked increase in vitamin E concentration in blood plasma samples was observed compared to the CON group. The duration of treatment influenced the concentration of vitamin E; in fact from the beginning to the end of the trial it was increased significantly in the three experimental groups, whereas in the CON group concentrations remained unchanged.

Table 2: Plasma oxidative markers in hares fed *Lippia citriodora* extract

Parameters	Experimental groups§				SEM¶	P-value‡		
	CON	LNE	MNE	HNE		D	T	D×T
Animals (n)	3	3	3	3				
ROMs (U.Carr^{-1})								
0 d	187.7	190.7^{a}	186.5^{a}	186.5^{a}	9.46			
240 d	199.9^{1}	137.1^{2b}	110.2^{3b}	122.4^{2b}	9.39	0.049	0.042	0.036
TBARS (mmol.L^{-1})								
0 d	0.162	0.152^{a}	0.166^{a}	0.171^{a}	0.034			
240 d	0.222^{1}	0.126^{2b}	0.128^{2b}	0.128^{2b}	0.048	0.004	0.042	0.045
Vitamin E (micr-mol.L^{-1})								
0 d	0.315	0.326^{a}	0.317^{a}	0.326^{a}	0.023			
240 d	0.299^{1}	0.367^{2b}	0.389^{2b}	0.455^{3b}	0.069	0.003	0.002	0.039
Vitamin A (micr-mol.L^{-1})								
0 d	0.252	0.267^{a}	0.278	0.275^{a}	0.017			
240 d	0.247^{1}	0.286^{2b}	0.285^{2}	0.332^{3b}	0.016	0.048	0.041	0.045

§ Control (CON); 1 g.kg^{-1} low natural extract (LNE); 1.5 g.kg^{-1} medium natural extract (MNE); 2 g.kg^{-1} high natural extract (HNE).
¶ SEM: Standard error of means
‡ D: fixed effect of dietary supplementation; T: fixed effect of time; D×T: interaction dietary supplementation x time.
^{1,2,3} Within a row, means without a common superscript differ ($P < 0.05$).
^{a,b} Within a column, means without a common superscript differ ($P < 0.05$).

The LNE, MNE and HNE groups showed a significant increase in the plasma concentration of vitamin A in LNE, MNE and HNE group, compared to the CON group. From the beginning to the end of the trial these values significantly increased in LNE and HNE groups, whereas in the CON and MNE group concentration remained unchanged. The increase in plasma vitamin A and E may be attributed to the ability of the NE to strengthen the endogenous antioxidant system. This is achieved by controlling the oxidative metabolism by reducing the production of reactive oxygen radicals and by inducing enzymes with antioxidant activities (Zhu *et al.,* 1999). Similar results were obtained in our previous studies on hare, on naturally milk-fed lambs and on ewes whose diet was supplemented with *Lippia* NE (Palazzo *et al.*, 2011; Casamassima *et al*., 2012, 2013a, 2013b).

Table 3 reported data on semen quality characteristics of hares, taken at 180 d, 210 d and 240 d.

All recorded data remained within the normality range of the species (Kozdrowski and Dubiel, 2005) and only the sperm motility values were affected by the dietary treatment.

At the end of the trial, sperm volume, pH and sperm concentration values were not affected by *Lippia* NE treatment, and the mean values recorded were 0.543 ml, 7.4 and 263.25 10^6 per ejaculate, respectively.

The dietary treatment negatively affected ($P < 0.05$) the sperm motility values in LNE, MNE and HNE groups, by 21.0 %, 19.7 % and 18.9 %, respectively. A time effect was observed in CON group with an increase of values by 9.9 %, and in HNE group, with a decrease by 14.9 % at the end of the experiment.

These results are in agreement with those of Vizzarri *et al*. (2010), where a possible negative effect of verbascoside (main bio-active component of *Lippia* NE) in rabbit quality semen was reported. In addition Dell'Aquila *et al*. (2014) observed a pro-oxidant effect of verbascoside on ovine prepubertal oocytes in *in vitro* experiment.

Unexpectedly, the dietary use of antioxidant supplement did not provide any improvement to the semen quality traits, as generally reported in literature (Yousef *et al.*, 2003), but it improved the welfare status of treated animals, as was reflected in a better lipid profile and oxidative markers.

A negative correlation coefficients were also reported between semen quality characteristics and AST and ALT enzyme activities (Yousef *et al*., 2003), but in our experiment no such correlation was found.

Table 3: Semen quality characteristics in hares fed *Lippia citriodora* extract

Parameters	Experimental groups§				SEM¶	P-value‡		
	CON	LNE	MNE	HNE		D	T	D×T
Animals (n)	3	3	3	3				
Sperm volume (ml)								
180 d	0.450	0.417	0.410	0.457[a]	0.017			
210 d	0.453	0.360	0.443	0.463[a]	0.024			
240 d	0.517	0.523	0.467	0.667[b]	0.036	0.470	0.001	0.076
pH								
180 d	7.27[1]	7.03[1]	8.13[2]	7.40	0.143			
210 d	7.40	7.10	8.10	7.43	0.207			
240 d	7.47	7.23	7.83	7.17	0.121	0.096	0.860	0.837
Sperm concentration (n 10^6/ejaculate)								
180 d	261.00	257.00	251.00	273.67	3.437			
210 d	264.00	261.33	242.67[1]	279.33[2]	7.693			
240 d	265.33	264.67	245.00	278.00	5.141	0.055	0.741	0.679
Sperm motility (%)								
180 d	70.67[a]	71.33	72.33	74.00[a]	0.883			
210 d	69.00[a]	62.33	61.00	61.33[b]	1.438			
240 d	77.67[1b]	61.33[2]	62.33[2]	63.00[2b]	2.169	0.028	0.001	0.001

§ Control (CON); 1 g.kg^{-1} low natural extract (LNE); 1.5 g.kg^{-1} medium natural extract (MNE); 2 g.kg^{-1} high natural extract (HNE).
¶ SEM: Standard error of means
‡ D: fixed effect of dietary supplementation; T: fixed effect of time; D×T: interaction dietary supplementation x time.
[1,2,] Within a row, means without a common superscript differ ($P < 0.05$).
[a,b] Within a column, means without a common superscript differ ($P < 0.05$).

CONCLUSION

In conclusion, the results of the present work underline a possible negative effect of the *Lippia* NE extract on the semen quality characteristics, besides the improvement in welfare status of the treated hares. Since a growing wide interest in dietary application of natural extract, further research is needed to assess the effect of a lower dose of *Lippia* extract on hare semen traits, taking in consideration the seminal plasma lipid peroxidation in correlation with blood oxidative markers.

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