PHYTODERIVATES IN RABBIT DIET AND IMMUNE RESPONSES

Daniela BEGHELLI[1]*, Raffaella CARDINALI[2], Sara CATERBI[3], Onelia BISTONI[3], Alessia ALUNNO[3], Roberto GERLI[3], Cesare CASTELLINI[2]

[1]School of Environmental Sciences. University of Camerino. Via Gentile III da Varano. 62032 Camerino, Italy
[2]Department of Applied Biology. Borgo XX Giugno, 74. University of Perugia. 06121 Perugia. Italy.
[3] Rheumatology Unit - Department of Clinical & Experimental Medicine. University of Perugia. Via del Giochetto. 06132 Perugia. Italy.
2 Mignini e Petrini spa, via dei Pini 3 06081 Petrignano d'Assisi Perugia, Italy
*Corresponding author∶daniela.beghelli@unicam.it

ABSTRACT

In order to investigate the effects of some dietary phytoderivates (*Origanum vulgare* L. and *Rosmarinus officinalis L.)* on rabbit peripheral lymphocyte proliferation and subsets populations, a total of 100 New Zealand mixed-sex rabbits were weaned at 30 days of age and immediately split into homogeneous groups submitted to the following dietary treatments: 1) Standard diet (Ctrl); 2) Standard diet +150 ppm Vit E (Vit E); 3) Standard diet +0.2% oregano (O); 4) Standard diet +0.2% rosemary (R) and 5) Standard diet +0,1% oregano + 0.1% rosemary (OR). Blood samples were collected from rabbits at 30 days (Time 0, n.10 rabbits) and 90 days of age (Time 1; n.5 animals/diet group). A diet effect on lymphocyte proliferation test was observed in the "O" group, where its response to pokeweed mitogen (PWM) and interleukin-2 (IL-2) at Time 1 resulted significantly higher than that of the other experimental diet groups (PWM, P<0,001 and IL-2, P< 0,000), with the exception of "OR" group whose IL-2 mitogen response was as significant higher as that of "O" group. Furthermore, the basal mitogenesis values revealed higher responses at Time 1 in the "Vit E" and "O", but the addition of mitogens induced significant increased responses only in the "O" group. No diet effects were registered on the lymphocyte subsets. The lymphocyte proliferation test in the control group showed a general reduction of leukocytes response with age, in particular it was observed a significant reduced response to PWM and PHA mitogens (P<0,021). Age effects were also recorded on lymphocyte subsets with a significant increase of CD8^{+}% with age (P<0,000). These results seem to encourage the integration of rabbit diet with oregano for its positive effect on adaptive immune response (a better *in vitro* reactivity of B cells).

Key words: Lagomorpha; dietary phytoderivates; immune responses

INTRODUCTION

Natural antioxidants are receiving increased attention in human and animal nutrition because of their association with food quality characteristics and immune response (Middleton and Kandaswami, 1992; Cullen *et al*., 2005). Among a variety of plants bearing antioxidative constituents, the Labiatae family (mint plants) have been attracting the greatest interest (Windisch *et al.,* 2008), with particular attention to products from oregano (*Origanum vulgare* L.) and rosemary (*Rosmarinus officinalis L.).* Oregano exert a well documented antioxidative activity (Economou *et al.*, 1991; Kokkini, 1994; Adam *et al.*, 1998; Cervato *et al*., 2000; Florou-Paneri *et al*., 2005), but it also possesses intense *in vitro* antimicrobial (Dorman and Deans, 2000) and antifungal (Daouk *et al*., 1995) properties, making it an appropriate candidate as a replacement for antibiotic growth promoters and also a promising food additive in order to prevent meat lipid oxidation (Symeon *et al*., 2010). Indeed, oregano has been shown to improve meat storage stability after slaughter in rabbits (Botsoglou *et al*., 2004). The rosemary extract exerts anti-oxidative activity, but its constituents have also shown a variety of pharmacological activities for cancer chemoprevention and therapy in *in vitro* and *in vivo* models (Faixsovà and Faix, 2008). The claim often made of phytogenic feed additives as stimulant of immune

function however faces with a poor specific experimental verification in monogastric animals, therefore there is a lack of data regarding the possible effects of plants extracts on immunity of these species. This study was designed to evaluate the 1) effect of dietary plant extract (oregano and/or rosemary), besides 2) age effect, on peripheral blood lymphocytes subsets and some specific immune responses (lymphocytes proliferation test *in vitro*) of rabbits.

MATERIALS AND METHODS

Animals

The animals were bred at the experimental facilities of the Dipartimento di Biologia Applicata of the University of Perugia. A total of 100 New Zealand mixed-sex rabbits were weaned at 30 days of age and immediately split into homogeneous groups submitted to the following dietary treatments: 1) Standard diet (Ctrl); 2) Standard diet +150 ppm Vit E (Vit E); 3) Standard diet +0.2% oregano (O); 4) Standard diet +0.2% rosemary (R) and 5) Standard diet +0.1% oregano + 0,1% rosemary (OR). Every diets contain a integration of 50 ppm Vit E, CLA 0.5% (from soy oil) + 3% Omega Lin ® (Mignini & Petrini) + 0.5% mixed vitamins. The plant derived ingredients were obtained with an enzyme aided extraction of leaves using water as solvent (Phenbiox). All rabbits were housed individually in flat-deck cages measuring 600 x 250 x 330 mm. Peripheral blood (**PB**) was obtained from the marginal ear vein of rabbits after washing of the ear with 70 per cent ethanol and collected into sterile heparinized tubes. Samples were collected from rabbits at 30 days (Time 0, n.10 rabbits) and 90 days of age (Time 1; n.5 animals/diet group). Samples were used for lymphocytes proliferation test, which provide estimates of one aspect of immune cell function, and to phenotyping the lymphocytes subsets. These assays were carried out at the S. Maria della Misericordia Hospital of Perugia in collaboration with the University of Camerino.

Lymphocyte Separation and proliferation test

Samples collected at Time O were analysed pooling the aliquots of two individuals (nearly 6 ml), whereas those of Time 1 pooling an aliquot of heparinized **PB** samples collected from five different rabbits of the same diet group (2 ml /each). **PB** was mixed with an equal volume of NaCl 0.9%. The mixture was laid on the top of 15 mL of Lhympholyte® (Cedarlane ®) in 50 ml sterile centrifuge tubes and centrifuged at 1600 rpm for 20 min at room temperature. The peripheral blood mononuclear cells (PBMC) layer was transferred to sterile culture tubes and washed twice with HBSS (Gibco®, Invitrogen^{TM}) without Ca^{2+} and Mg^{2+}. Then the cells were suspended in complete RPMI-1640 medium (Euroclone®) that contained fetal bovine serum (10%; Gibco®, Invitrogen^{TM}), L-Glutammine (2 mM; Euroclone®), penicillin (100 U/mL; Biochrom^{AG}, Berlin), and streptomycin (100 μg/mL; Biochrom^{AG}, Berlin). The number of live lymphocytes was determined using an automatic haemocytometer and a trypan blue dye exclusion procedure (Countess, Invitrogen^{TM}). The final concentration of live cells was adjusted to 2×10^6/mL of complete medium. Into each well of flat bottom 96-well tissue culture plates (Becton Dickinson, Lincoln Park, NJ), PBMC suspension (2×10^5 live cells) of each pool sample were cultured in. Mitogenesis was stimulated with concanavalin A (**Con A**; stimulates T-lymphocytes; 10 μg/ml; Sigma. Only for Time 1 samples), pockedweed mitogen (**PWM**, stimulates B-lymphocytes; 0.5 μg/well; Sigma), phytohaemagglutinin (**PHA**; 240μg/ml; Biochrom^{AG}, Berlin, stimulates T-lymphocytes), interleukin-2 (**IL-2**, 1U/ml; Novartis, Italy stimulates B- and T-lymphocytes) or it was not stimulated (100 μL of Medium/well) so that the basal mitogenesis could be estimated. Incubation treatments were in triplicate. The plates were held at 37°C for 120 h in a humidified chamber with an atmosphere of 5% CO2 in air. Then, 1 mCi of [3H]thymidine (specific radioactivity 4 Ci/mmol; PerkinElmer,) in RPMI-1640 was added to each well, and the plates were held under the same conditions for another 16-18 h. At the end of culture, the cells were mashed and transferred to filter discs corresponding to each well. These filter discs were soaked in scintillation liquid (Betaplate SCINT; PerkinElmer), and the lymphocytes were solubilized to release the [3H]thymidine. Disintegrations per minute (cpm) were determined with a liquid scintillation counter (Trilux 1450

Microbeta, Wallac) and used to calculate the picomoles of [3H]thymidine incorporated into newly synthesized DNA.

Peripheral staining and flow cytometry

Commercially available mAbs were used for the detection of lymphocyte subsets in heparinized **PB** samples collected from five different rabbits of each diet groups (5 groups). The surface staining of blood leukocytes was performed using PE, FITC labeled anti-rabbit $CD4^+$ (KEN-4 clone, AbD Serotec) and $CD8^+$ (12.C7 clone, AbD Serotec) T-cells-specific antigen; whereas, APC labelled mouse anti-human $CD79^+$ (clone HM47, BD, eBioscience and BioLegend), cross-reactive antibody against rabbit B^+-cells antigen was used for intracellular staining. The primary FITC anti-rabbit $CD8^+$ mAb was diluted 1:10 in phosphate buffer solution (**PBS**). 50 μl of **PB** was incubated at 4°C for 20 min in 12x75 mm flow cytometry tubes with a combination of the two primary mAbs (antiCD4/antiCD8; 5 μl, respectively), after incubation cells were washed twice in PBS and the erythrocytes were lysed by 2 ml of hemolytic solution (8,26 g NH_4Cl, 1 g $KHCO_3$ and 0,037 EDTA per 1 L of distilled water). Flow cytometric analysis of these primary mAbs was performed using a standard FACSCalibur^{TM} flow cytometer (Becton Dickinson, Mountain View, CA) operated by the CELLQuest^{TM}software. In each sample, 10.000 cells were measured and the data were saved in the list mode. The same samples were finally permeabilized with 0.1% saponin blocking buffer after 4% paraformaldehyde fixation to label $CD79^+$ cells (APC^+ rabbit B-lymphocytes) according to a protocol recommended by the produces, and data acquired again by flow cytometer. Gating was based on forward angle and right angle scatter signals.

Statistical analysis

One way ANOVA for both the phenotyping data and lymphocytes proliferation test was done, with a Bonferroni and Dunnet's Post Hoc multiple comparisons test applied to the significant variables between the diet groups (SPSS 13 statistical package).

RESULTS AND DISCUSSION

An effect of "age" (Ctrl group at Time 0 *vs* Time 1: $CD8^+$%, P<0.000) (data not shown) was seen in the leukocyte phenotype subsets of **PB** sample. Furthermore, the $CD8^+$leukocytes subsets percentages individually evaluated in all the experimental diet groups at Time 1 *vs* those recorded at Time 0 maintained the significant difference (Table 1), with a further "age" effect recorded in the "R group" for the $CD79^+$ subset (Time 0 *vs* Time 1; P<0.024) (Table 1). However, no significant diet effect was observed for the **PB** lymphocyte subset percentages analyzing data exclusively recorded at Time 1.

Table 1: Effect of age within the diet groups on PB leukocytes phenotype subsets (expressed as %).

	Time 0 (Ctrl)	Time 1 (Ctrl)	Time 1 (Vit E)	Time 1(O)	Time 1 (R)	Time 1 (OR)
$CD4^+$(%)	16.3 ± 5.7	17.7 ± 3.2	16.6 ± 2.4	17.1 ± 2.3	14.5 ± 3.7	21.2 ± 5.7
$CD8^+$(%)	26.5^{Aa} ± 2.7	37.9^B ± 4.1	37.1^B ± 4.7	38.0^B ± 4.7	35.0^b ± 5.8	40.3^B ± 4.1
$CD4^+CD8^+$(%)	1.2 ± 0.5	1.1 ± 0.4	0.9 ± 0.3	1.1 ± 0.3	0.8 ± 0.4	0.7 ± 0.2
$CD79^+$(%)	9.0^a ± 4.1	13.6^{ab} ± 3.7	12.1^{ab} ± 2.7	12.6^{ab} ± 3.5	15.2^{bc} ± 3.5	13.1 ± 3.5

^{A,a}: different letter denotes significant difference, P<0.005 or< 0.05, respectively. Data are given as the mean ± Standard Deviation

The lymphocyte proliferation test showed a general reduction of leukocytes response with age, in particular it was observed a significant reduced response to PWM and PHA mitogens (P<0.021; data not shown). Whereas, a diet effect on lymphocyte proliferation test was observed in the "O" group, where its response to PWM and IL-2 at Time 1 resulted significantly higher than that of the other treatment groups (PWM, P<0.001 and IL-2, P< 0.000), with the exception of "OR" group whose IL-2 mitogen response was as significant higher as that of "O" group. Furthermore, the basal mitogenesis

values revealed (cells cultured without mitogen) higher responses at Time 1 in the "Vit E" and "O" (data not shown), but the addition of mitogens induced significant increased responses only in the "O" group.

Table 2: Effect of diet on lymphocyte proliferation test evaluated at Time 1 and expressed as % of cpm increase.

	% cpm increase Ctrl	% cpm increase Vit E	% cpm increase O	% cpm increase R	% cpm increase OR	P<
PWM	$0.31^B \pm 0.3$	$0.6^B \pm 0.5$	$2.5^A \pm 0.6$	$0.4^B \pm 0.3$	$1.3^{AB} \pm 0.3$	0.001
PHA	0.7 ± 1.2	0.9 ± 0.3	0.3 ± 0.6	0.8 ± 0.6	0.9 ± 0.6	n.s.
IL-2	$0.2^B \pm 0.4$	$2.2^B \pm 0.8$	$16.4^A \pm 4.3$	$0.4^B \pm 0.4$	$5.4^A \pm 0.5$	0.000
ConcA	1.5 ± 1.5	0.0 ± 0	0 ± 0	0.8 ± 0.2	0.3 ± 0.3	n.s.

[A,B..]: different letter denotes significant difference, P<0.001. Data are given as the mean ± Standard Deviation

There are very little data concerning the immune response of rabbits. Here we observed an age effect on the normal expression of lymphocyte subsets, with an significant increase of the $CD8^+$ % with age. It seems that the immune response mediated by cytotoxic T cells ($CD8^+$) becomes more relevant with age (Table 1). In these rabbits the presence of a **PB** $CD4^+CD8^+$T cell population has been stated and although very little is known about **PB** $CD4^+CD8^+$T cells role in immune response *in vivo,* several observations in humans, chicken and porcine suggest that peripheral $CD4^+CD8^+$T cells function as normal T cells and respond to signals delivered by mitogens. Interestingly, in contrast to that observed in pigs and humans where the number of **PB** $CD4^+CD8^+$T cells increase or decrease, respectively, with age, in our rabbits they seem to be not varying during their productive life. Furthermore, several studies demonstrate that also an intestinal $CD4^+CD8^+$T cell population is present in all individuals and although of different origin, it has been suggested that the same mechanisms (age or the presence of gastrointestinal flora) could still result in a co-expression of $CD4^+$ and $CD8^+$ both on **PB** and intestinal T cell populations (Luthala, 1998). In this study a diet supplemented of phytoderivates, that could also affect the gastrointestinal flora, doesn't seem to have affected **PB** CD4+CD8+ T cells population. Whereas, the "O" diet influenced the adaptive immune response (T and B cells) with a better *in vitro* reactivity of B cells (stimulate both by PWM and IL-2 mitogens). The "OR" group gathered intermediate results (blastogenic response to IL-2, P<0.000; Table 2) that could be expression of the "positive oregano contribute" to the "OR" diet. This promising results will be tested in a second experimental trial to confirm these data and other variables will be considered to try to better understand how the oregano exerts its modulating effect on the immune response.

ACKNOWLEDGEMENTS

REFERENCES

Adam K., Sivropoulou A., Kokkini S., Lanaras T., Arsenakis M. 1998. Antifungal activities of *Origanum vulgare* subsp. *hirtum*, *Mentha spicata*, *Lavandula angustifolia*, and *Salvia fruticosa* essential oils against human pathogenic fungi. *J. Agri. Food Chem., 46, 1739-1745.*

Botsoglou N.A., Florou-Paneri P., Christaki E., Giannenas I., Spais A.B. 2004. Performance of rabbits and oxidative stability of muscle tissues as affected by dietary supplementation with oregano essential oil. *Arch. Anim. Nutr., 58, 209-218.*

Cervato G., Carabelli M., Gervasio S., Cittera A., Cazzola R., Cestaro B. 2000. Antioxidant properties of oregano (origanum vulgare) leaf extracts. *J. Food Biochem., 2, 453-465.*

Cullen S.P., Monahan F.J., Callan J.J., O'Doherty J.V. 2005. The effect of dietary garlic and rosemary on grower-finisher pig performance and sensory characteristics of pork. *Irish J. Agric. Food Res.,* 44, 57–67.

Daouk, R.K., Dagher S.M., Sattout E.J. 1995. Antifungal activity of the essential oil of *origanum syriacum* L. *J. Food Prot., 58, 1147-1149.*

Dorman H.J.D. and Deans S.G. 2000. Antimicrobial agents from plants: Antibacterial activity of plant volatile oils. *J. Appl. Microbiol., 88, 308-316.*

Economou, K.D., Oreopoulou V., Thomopoulos C. 1991. Antioxidant properties of some plant extracts of the Labiatae family. *J. Am. Oil Chem. Soc., 68, 109-113.*

Faixová Z. and Faix S. 2008. Biological effects of rosemary (*Rosmarinus officinalis L.*) essential oil. *Folia Veterinaria.*, 52, 3-4, 135-139.

Florou-Paneri P., Palatos G., Govaris A., Botsoglou D., Giannenas I., Ambrosiadis I. 2005. Oregano Herb Versus Oregano Essential Oil as Feed Supplements to Increase the Oxidative Stability of Turkey Meat. *Int. J. Poultry Sci., 4 (11,: 866-871.*

Luthala M. 1998. Chicken CD4, CD8ab, and CD8aa T Cell Co-Receptor Molecules *Poultry Sci., 7, 1858–1873.*

Kokkini S. 1994. Herbs of the *Labiatae*. In R. Macrae, R. Robinson, M. Sadler, and G. Fuulerlove (Eds.), Encyclopaedia of Food Science, Food Technology and Nutrition, pp: 2342-2348, London, Academic Press.

Middleton E.Jr and Kandaswami C. 1992. Effects of flavonoids on immune and inflammatory cell functions. *Biochem. Pharmacol. 43, 613-619.*

Symeon G.K., Zintilas C., Demiris N., Bizelis I.A., Deligeorgis S.G. 2010. Effects of oregano essential oil dietary supplementation on the feeding and drinking behaviour as well as the activity of broilers. *Int. J. Poultry Sci., 9 (4), 401-405.*

Windisch W., Schedle K., Plitzner C., Kroysmayr A. 2008. Use of phytogenic products as feed additives for swine and poultry. *J. Anim. Sci., 86, E140-E148.*