PHYTODERIVATES IN RABBIT DIET AND IMMUNE RESPONSES

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ABSTRACT

In order to investigate the effects of some dietary phytoderivates (Origanum vulgare L. and Rosemarinus 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 (Rosemarinus 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 phyto-genic 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 0 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 Lympholyte® (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®, InvitrogenTM) without Ca²⁺ and Mg²⁺. Then the cells were suspended in complete RPMI-1640 medium (Euroclone®) that contained fetal bovine serum (10%; Gibco®, InvitrogenTM), L-Glutamine (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, InvitrogenTM). The final concentration of live cells was adjusted to 2 x10⁶/mL of complete medium. Into each well of flat bottom 96-well tissue culture plates (Becton Dickinson, Lincoln Park, NJ), PBMC suspension (2 x10⁵ 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), pockeedweed 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 (Betalapia 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\(^+\) (12C7 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 \(\mu\)l 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 \(\mu\)l, respectively), after incubation cells were washed twice in PBS and the erythrocytes were lysed by 2 ml of hemolytic solution (8,26 g NH\(_4\)Cl, 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\(^\text{TM}\) flow cytometer (Becton Dickinson, Mountain View, CA) operated by the CELLQuest\(^\text{TM}\) 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 %).

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

\(^a,b,c\): 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.

<table>
<thead>
<tr>
<th></th>
<th>% cpm increase</th>
<th>% cpm increase</th>
<th>% cpm increase</th>
<th>% cpm increase</th>
<th>% cpm increase</th>
<th>P&lt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ctrl</td>
<td>Vit E</td>
<td>O</td>
<td>R</td>
<td>OR</td>
<td></td>
</tr>
<tr>
<td>PWM</td>
<td>0.31 ± 0.3</td>
<td>0.60 ± 0.5</td>
<td>2.50 ± 0.6</td>
<td>0.40 ± 0.3</td>
<td>1.30 ± 0.3</td>
<td>0.001</td>
</tr>
<tr>
<td>PHA</td>
<td>0.7 ± 1.2</td>
<td>0.9 ± 0.3</td>
<td>0.3 ± 0.6</td>
<td>0.8 ± 0.6</td>
<td>0.9 ± 0.6</td>
<td>n.s.</td>
</tr>
<tr>
<td>IL-2</td>
<td>0.20 ± 0.4</td>
<td>2.20 ± 0.8</td>
<td>16.40 ± 4.3</td>
<td>0.40 ± 0.4</td>
<td>5.40 ± 0.5</td>
<td>0.000</td>
</tr>
<tr>
<td>ConcA</td>
<td>1.5 ± 1.5</td>
<td>0.0 ± 0.0</td>
<td>0 ± 0</td>
<td>0.8 ± 0.2</td>
<td>0.3 ± 0.3</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

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+CD8+ both on PB and intestinal T cell populations (Luthala, 1998). In this study a diet supplemented of phytodervatives, 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.

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