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Effects of oregano (*origanum vulgare l*) and rosemary (*rosmarinus officinalis l*) aqueous extracts on *in vitro* rabbit immune responses

Abstract

In order to investigate the effects of some dietary phytoderivates (*Origanum vulgare L.* and *Rosmarinus officinalis L.)* on rabbit immune system, 100 New Zealand mixed-sex rabbits weaned at 30 days of age were split into homogeneous groups submitted to the following dietary treatments: 1) Standard diet (C); 2) C +150 ppm Vit E (E); 3) C +0.2% oregano (O); 4) C +0.2% rosemary (R) and 5) C +0.1% oregano + 0.1% rosemary (OR). Blood samples were drawn from rabbits at 30 and 90 days of age (ten rabbits/diet group at Time 0 and 1, respectively) to investigate the blood lymphocyte subset evolution, the *in vitro* lymphocyte proliferation (in presence or absence of mitogens) responses and the Immunoglobulin M (IgM) concentration in the supernatants of lymphocyte cultures. A diet effect was observed in the O and OR groups, where the lymphocytes proliferation responses to pokeweed mitogen (PWM; $^{***}P<0.001$, in the O group) or the interleukin-2 production (IL-2; $^{***}P<0.001$, for both groups) at Time 1 were significantly higher. The IgM levels were systematically higher in the O, OR and E cells culture supernatants ($^{**}P<0.01$). Age did not affect the rabbit lymphocyte subset evolution nor the *in vitro* lymphocyte proliferation. Data obtained in the present study show that rabbit's dietary supplementation with oregano elicits positive effects on the adaptive immune response.

Keywords: dietary phytoderivates, flow cytometry, igm, t cells, lymphocyte proliferation assay, rabbit, immune function, animal nutrition

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Abbreviations: PB: Peripheral Blood; mAbs: Monoclonal Antibodies; HBSS: Hank's Balanced Salt Solution; PBMC: Peripheral Blood Mononuclear Cell; PWM: Pokeweed Mitogen; GALT: Gut-Associated Lymphoid Tissue

Introduction

Natural antioxidants are receiving increasing attention in human and animal nutrition because of their association with food quality characteristics and immune responses.[1,2] Among a variety of plants bearing anti-oxidative constituents, the Labiatae family (mint plants) has been attracting the greatest interest.[3] with particular attention to products from oregano (*Origanum vulgare L.*) and rosemary (*Rosmarinus officinalis L.).* Oregano exerts a well documented anti-oxidative activity.[4-6] but it also possesses intense *in vitro* antimicrobial.[7] and antifungal.[8] properties. Such properties make it an appropriate candidate as a replacement for antibiotic growth promoters and also a promising food additive in order to prevent meat lipid oxidation.[9] Indeed, oregano has been found to improve meat storage stability after slaughter in rabbits.[10] 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.[11] The claim often made of phytogenic feed additives as stimulant of immune function however faces with a poor specific experimental verification in monogastric animals (Lagomorpha). 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: 1) the effect of dietary plant extract (oregano and/or rosemary) and 2) age effect, on some specific immune responses (lymphocytes proliferation test and IgM measurements *in vitro*) and on peripheral blood lymphocyte subsets development in rabbits.

Materials and methods

Animal dietary treatment and blood sampling

The animals were bred at the experimental facilities 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 (C); 2) C + 150 ppm Vit E (E); 3) C + 0.2% oregano (O); 4) C + 0.2% rosemary (R) and 5) C + 0.1% oregano + 0.1% rosemary (OR). Every diets contained an integration of 50ppm 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, Calderara di Reno, Bologna). All rabbits were individually housed in flat-deck cages measuring 600 x 250 x 330 mm. Peripheral blood (PB) samples were taken from rabbits at 30 and 90 days of age (ten rabbits/diet group at Time 0 and 1, respectively). These samples were drawn from the marginal ear vein after washing with a 70% ethanol solution, using vacuum heparinized tubes. Diurnal variations in hematological parameters were minimized by collecting blood at approximately the same time (8:00-10:00 am). Blood samples were processed within 1h after sampling.

Lymphocyte labeling and flow cytometry analysis

Commercially available monoclonal antibodies (mAbs) were used for the detection of lymphocyte subsets. The surface staining of blood leukocytes was performed using a PE labeled mouse anti-rabbit $CD4^+$ mAb (KEN-4 clone, AbD Serotec/Bio-Rad Laboratories Inc., Segrate, Milan) or a FITC labeled mouse anti-rabbit $CD8^+$ mAb (12.

C7 clone, AbD Serotec/Bio-Rad Laboratories Inc., Segrate, Milan), both recognizing T-cells-specific antigen. An APC labeled mouse anti-human CD79α$^+$ mAb (clone HM47, BD, eBioscience Inc. and BioLegend, San Diego) a cross-reactive antibody against rabbit B$^+$ cells antigen were used for intracellular staining.[12] Flow cytometry analysis was performed using a standard FACSCalibur flow cytometer (Becton Dickinson, Lincoln Park, NJ) operating the Cell Quest Pro™ software. In each sample, 10.000 cells were measured and the data were saved in the list mode. Gating was based on forward angle and right angle scatter signals. The gates of each leucocyte type were adjusted with an isotype negative control. After a 4% paraformaldehyde fixation, the same samples were permeabilized with 0.1% saponin blocking buffer for intracellular staining with APC labeled CD79α$^+$ mAb according to manufacturer's instructions and data were again acquired by flow cytometer. Up to three different fluorochromes were analysed in the same vial.

Lymphocyte proliferation test

The lymphocyte proliferation test was performed pooling blood aliquots (2 mL/each) of two individual samples. Mixture of equal volumes of blood samples and NaCl 0.9% were layered on the top of 15 mL of Lympholyte (Cedarlane® Burlington, North Carolina) and centrifuged at 400 x g for 20 min at room temperature. The peripheral blood mononuclear cell (PBMC) layer was then transferred to sterile culture tubes and washed twice with Hank's Balanced Salt Solution (HBSS) (Gibco Invitrogen, Thermo Fischer Scientific Inc,) without Ca^{2+} and Mg^{2+}. Then the cells were re-suspended in complete RPMI-1640 medium (EuroClone S.p.A., Milan) that contained fetal bovine serum (10%; Gibco, Invitrogen), L-Glutammine (2 mM; Euroclone), penicillin (100 U/mL; Biochrom^{AG}, Berlin), and streptomycin (100 µg/mL; Biochrom^{AG}). The number of live lymphocytes was determined using an automatic haemocytometer and a trypan blue dye exclusion procedure (Countness, Invitrogen,). The final concentration of live cells was adjusted to 2 x10^6/mL of complete medium. PBMC suspension (2 x10^5 live cells) of each pool sample was cultured in flat bottom 96-well tissue culture plates (Becton Dickinson). Cells were stimulated with pokeweed mitogen (PWM) (that stimulates B$^+$ lymphocytes; 0.5 µg/well; Sigma Aldrich Co Ltd, Saint Louis , Missouri), phytohaemagglutinin (PHA; 1.2 µg/mL; Biochrom^{AG}), IL-2 (that stimulates B$^+$ and T$^+$ lymphocytes; 1U/mL; Novartis, Basilea). Cell cultured only with medium (100 µL/well) allowed to estimate the basal proliferation.[13]. Each culture condition was performed in triplicate. The plates were held at 37 °C for 72 h in a humidified chamber with an atmosphere of 5% CO_2 in air. Then, 1 mCi of.^{3H} thymidine (specific radioactivity 4 Ci/mmol, PerkinElmer, Waltham, Massachussetts) in RPMI-1640 was added to each well, and the plates were held under the same conditions for 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 were determined with a liquid scintillation counter (Trilux 1450 Microbeta, Wallac, PerkinElmer) and used to calculate the picomoles of.^{3H} thymidine incorporated into newly synthesized DNA. The results are expressed as counts per minute (cpm). The increase of lymphocyte proliferation due to the mitogenic agents adding was expressed as percentage increase of cpm (M cpm) *vs* basal cpm values (C cpm; only medium). Therefore, the applied formula was: increased proliferation = (M cpm- C cpm)/ C cpm, and it was expressed as %.

Titration of igm in the culture supernatants

Before the addition of.^{3H} thymidine, but only in samples collected at Time 1, 100 µL/well of supernatants were collected from each triplicate of each experimental condition and pooled. Then 100 L/well were transferred to new flat bottom 96-well tissue culture plates (Becton Dickinson) and promptly frozen at -20 °C until analysis. The rabbit IgM released in culture media were determined by a commercially available ELISA kit (Cat. N. E120-110-23, Bethyl Laboratories, Montgomery) and an automated washing and reader instrument (Mago4S, Diamedix Corporation, Hialeah) at 450 nm wavelength. The procedures for sample assays were carried out according to manufacturer's instructions.

Statistical analysis

Differences between dietary groups were assessed by ANOVA test with a Bonferroni and Dunnet's Post Hoc multiple test applied for comparison of all pairs when the critical assumptions for the independent-samples *t* test were valid. In the other cases, nonparametric tests for two or multiple independent samples were used (Mann-Whitney and Kruskall-Wallis tests). Differences with *p* value at least <0.05 were considered statistically significant. Due to the relatively small numbers of experimental animals, age-dependent changes were tested with the one-tailed Mann-Whitney non parametric test (all the rabbits of Time 0 *vs* those of the C group at 90 days). All calculations were performed with SPSS software (2004).[14]

Results

Lymphocyte subsets development in growing rabbits feed with different diets

No age-related changes in relative PB lymphocyte subsets were observed from 30 to 90 days of age in young adult rabbits; whereas a diet effect was observed for CD8$^+$ cell subpopulation at 90 days of their life (Table 1). Indeed, rabbits fed with the R diet showed lower CD8$^+$ percentages compared to all other experimental groups with values that become significant in comparison to the OR group (Table 1, $^*P<0.05$).

It is noteworthy that the sum of PB lymphocytes CD4$^+$ with CD8$^+$, CD4$^+$CD8$^+$, and CD79α$^+$ during the experimental period was always lower than 100% (by about 19.3 to 29.4%), these cells, according to Jeklova et al.[12] are referred to as lymphocytes with a CD4$^-$, CD8$^-$, and CD79α$^-$ phenotype.

Lymphocyte proliferation assay results

The rabbit lymphocyte proliferation test showed a general reduction of leukocytes response from 30 to 90 days of age, in particular for IL-2, by 3.8% ± 3.9 to 0.04% ± 0.06; for PHA, by 6.2% ± 6.7 to 0.7% ± 1.3; and finally, for PWM by 0.4% ± 0.5 to 0.3% ± 0.3 (Mann-Whitney test). However, the lower lymphocyte *in vitro* responsiveness was never statistically significant.

The rabbit lymphocyte proliferation assay responses, conversely, were influenced by experimental diets; indeed, at Time 1, the O group responses resulted significantly higher to PWM and IL-2 mitogens adding than those of the other diets groups ($^{***}P<0.001$) with the exception of OR group whose response to IL-2 was as significant higher as that of O group (Table 2). Although the proliferation of rabbits PBMCs cultured in absence of mitogens revealed higher responses in the "E" and "O" groups (data not shown), the addition of mitogenic agents induced significant increased responses only in those groups where animals were supplemented with oregano.

Table 1 PB lymphocyte subsets distribution (%) in different rabbits age or diet groups (mean ± SD)

	Time 0(C)	Time 1(C)	Time 1(E)	Time 1(O)	Time 1(R)	Time 1(OR)
$CD4^+$(%)	41.6 ± 8.5	38.1 ± 4.2	37.7 ± 4.7	39.1 ± 5.0	36.2 ± 5.4	41.5 ± 4.2
$CD8^+$(%)	16.8^{ab}± 2.4	17.8^{ab}± 3.2	16.8^{ab}± 2.4	17.5^{ab}± 2.4	15.0^{ac}± 3.7	22.0^{b}± 5.5
$CD4^+CD8^+$(%)	1.6 ± 0.6	1.6 ± 0.7	1.3 ± 0.5	1.5 ± 0.3	1.3 ± 0.5	1.1 ± 0.2
$CD79^+\alpha$(%)	20.7 ± 7.1	17.7 ± 5.6	14.8 ± 4.4	16.7 ± 4.0	20.3 ± 2.3	14.8 ± 3.8
CD4/CD8 (ratio)	2.2 ± 0.4	2.2 ± 0.4	2.3 ± 0.6	2.3 ± 0.5	2.5 ± 0.7	2.0 ± 0.8

[a,b,c]Means not sharing the same superscript being significantly different at $^*P < 0.05$ (one way ANOVA, Bonferroni Post hoc test).

SD= Standard Deviation.

Table 2 Effects of dietary phytoderivates (O: oregano; R: rosemary; OR: both oregano and rosemary) on rabbits lymphocyte proliferation test, at Time 1 (mean ± SD)

	Experimental Diet Groups				
Mitogens[a]	**C**	**E**	**O**	**R**	**OR**
PWM (%)[b]	0.31^{B} ± 0.3	0.6^{B} ± 0.5	2.5^{A} ± 0.6	0.4^{B} ± 0.3	1.3^{AB} ± 0.3
PHA (%)	0.7 ± 1.2	0.9 ± 0.3	0.3 ± 0.6	0.8 ± 0.6	0.9 ± 0.6
IL-2 (%)[c]	0.2^{B} ± 0.4	2.2^{B} ± 0.8	16.4^{A} ± 4.3	0.4^{B} ± 0.4	5.4^{A} ± 0.5

[*A,B,]Means not sharing the same superscript being significantly different at $^{***}P < 0.001$ ([b]Bonferroni test; [c]Tamhane test).

[a]Values are expressed as percentage of increased cpm *vs* basal cpm values (see paragraph 2.3).

SD= standard deviation.

IgM in the rabbit lymphocytes culture supernatants

The titration of rabbit IgM in culture supernatants revealed a strong effect of diets ($^{***}P<0.001$), with the highest Ig values recorded in the O group (608.6 ± 28.2 ng/mL) followed by OR (578.4 ± 33.2 ng/mL), E (560.7 ± 23.7 ng/mL), C (461.9 ± 28.2 ng/mL) and R (218.5 ± 24.2 ng/mL) groups. The mitogens added to the cells cultures did not particularly affect the IgM levels, indeed in some diet groups their higher values were just observed in supernatants of cells cultured without any stimulus (Figure 1). However, independently of the presence of mitogens, the O group always showed the highest values, followed by the OR group.

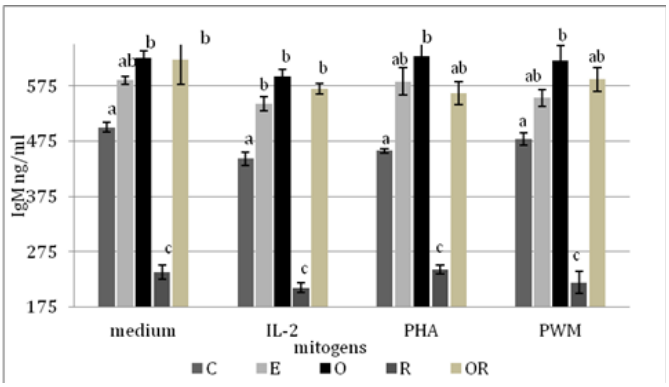

Figure 1 IgM levels in the rabbits lymphocytes culture supernatants, represented according to the diet groups (C, E, O, R, and OR) and mitogens (only medium, IL-2, PHA, and PWM.

[a-c]Means not sharing the same superscript being significantly different at $^{**}P < 0.01$ (Bonferroni test).

Discussion

Rabbits have been traditionally used as experimental models in human and veterinary research for many years, however, little is known about their blood lymphocyte populations or immune responses *in vitro*.[12,13,15-21]

In this study, the lymphocyte subsets were not influenced by animal's age and the relative numbers of $CD4^+$ or $CD8^+$ T cells were in accordance with those reported by some authors (calculated by Guerrero et al.[20]) or slightly higher than those reported by others.[12,22] However, in rabbits, T helper lymphocytes prevail on cytotoxic T cells that, conversely, represent a minor subset in their T-lymphoid compartment. The CD4/CD8 ratio was also substantially constant over time. The mechanism determining the blood peripheral CD4/CD8 ratios seem to be species-specific, originates in the thymus and is genetically controlled.[23,24] There are controversial observations related to an increase or a decrease in CD4/CD8 ratio during maturation. A reduction in the value of this ratio seems to be a common age-related event in mammals and it was observed in mice.[25] cats.[26] dogs.[27] pigs.[28] and rabbits.[12] However, it has been shown to possibly increase or decrease with age in humans.[29-32] Although Jeklova et al.[12] reported a rabbit CD4/CD8 ratio decreasing with age, the value they obtained from adult rabbits (20- week-old) is even slightly higher than that reported here at 90 days of age (2.8 ± 0.8 *vs* 2.2 ± 0.4 of the C group, Table 1). Furthermore, their value was obtained comparing 1-day-old rabbits to the adult ones. The animals studied here have been examined for a shorter period of time and they were slightly older at the beginning of the study, maybe this can explain why we did not observe a reduction in the ratio.

The relative number of double positive (DP) lymphocytes ($CD4^+CD8^+$ T cells) was found to be comparable to other reports, while the $CD79\alpha^+$ B cells were lower (16.9 ± 0.8 *vs* 42.5 ± 4.7, respectively) (12, pp. 636). However, our data seems to be quite similar to those calculated by the absolute values presented in the Ferrian et al.[21]

Very little is known about DP $CD4^+CD8^+$ T cell role in immune response *in vivo*. Several observations in humans, chickens and pigs suggest that peripheral $CD4^+CD8^+$ T cells function as normal T cells, respond to signals delivered by mitogens and may consist of memory cells; in humans, the presence of these cells in inflammatory sites, lamina propria of the gut and kidney allografts suggest an immunoregulatory and/or immunosurveillance function.[33,34] According to other authors.[35] in pigs the DP T lymphocytes could also be defined as memory/activated $CD4^+CD8^+$T cells (T_{helper}). However,

in contrast to what was observed in pigs, monkeys and chickens, where the number of PB $CD4^+CD8^+$ T cells increase with age, in this study we did not observe any changes in their percentages and this is in accordance with other studies.[12]

Although the role of environmental (microbial and feed) antigens on the generation of DP lymphocytes has not been thoroughly examined in many species, exposure to antigens appears to play a role in the creation of DP cells both *in vitro* and *in vivo*.[34]

Several studies demonstrated that also an intestinal DP $CD4^+CD8^+$ T cell population is present in birds and mammals.[33] and although of different origin, it has been suggested that the same mechanisms (age or antigen exposure in the intestine/gastrointestinal flora) could induce a co-expression of $CD4^+$ and $CD8^+$ both on PB and on intestinal T cell populations.[33] However, in this study even if the diet supplemented with phytoderivates (oregano and/or rosemary) had affected the gastrointestinal flora, its effect had not been strong enough to influence the DP $CD4^+CD8^+$ T cells population in blood. A possible immunoregulatory role of phytoderivates may have been better observed in conventional rabbit herds with a major antigenic exposure (instead of these controlled conditions) or in older rabbits.

The lowest $CD8^+$ cell values observed in R group (significantly lower than in OR group, although within the rabbit range, Table 1) could express worse responses to signals from microbial environment, particularly from the commensal microflora of the gastrointestinal and respiratory tracts, that usually are responsible for the post-natal maturation of the immune functions.[36]

A possible explanation for the lack of effectiveness of rosemary in positively modulating the rabbits immune responses *in vitro*, besides a lower % of $CD8^+$ cells at 90 days of age (although it possesses known antioxidant properties that exert a positive effect on immune functionality; 15), as already obtained in rats.[37] could be found in the use of an incorrect concentration of rosemary administered. Finally, the non-detected cells to reach 100% of PBMCs (in this study between 19.3-29.4%), previously referred to as lymphocytes with $CD4^-$, $CD8^-$, and $CD79\alpha^-$ phenotype, may be a γδ T cell subpopulation as suggested by some authors.[12] Indeed, in contrast to human and mice, rabbits together with cattle, sheep, pigs and chicken belong to species with sizeable γδ peripheral T cell pool.[38] about 23% of $/\delta^+$ PB T-cell for some authors.[22] or 16-48% for others.[12,20] In the first study these cells were individuated by using an anti-human TCR- γ/δ^+mAb, in the others these numbers were obtained/deducted by the subtraction of lymphocytes subsets recognized by $CD4^+$, $CD8^+$, and $CD79\alpha^+$ mAbs (or a mouse anti-rabbit α-pan B) from their total number. The current difficulty for immunological research into rabbits lies in the still limited number of commercially available monoclonal antibodies that recognize the various lymphocyte subsets antibodies.[39] between these an anti-rabbit γδ mAb.

If to date there are limited data available in literature on rabbit lymphocyte phenotyping, those relative to the rabbit *in vitro* mitogen-induced PB lymphocyte proliferation assays are even scarcer.[13] and, to the best of our knowledge, none concerning IgM titration. In this study, we observed an age-related reduction of rabbit lymphocyte proliferation responses that seems to be in contrast with what reported by other authors.[40] However, these authors referred to depressed immunologic capabilities in splenic lymphocytes of newborn rabbits; whereas the same observed a restored immune functionality over the initial 2 to 6 weeks of life. To our knowledge, there are no other studies to refer to, but since our observations began at the fourth week of rabbit life and on a lymphocyte population of a distinct district (PB) it is quite difficult to make comparisons with the aforementioned paper results. The influence of intestinal microflora on diversification of primary antibodies and selection of B cells has been intensively studied.[12,41,42] Rabbits, like chickens but unlike other species, require environmental stimuli for somatic diversification of the antibody repertoire, in particular the primary antibody repertoire that develops between 3 and 8 weeks of age. The exogenous factors that act as stimuli apparently derived from the gut microbial flora.[42] and cause a somatically diversifying of the neonatal repertoire through somatic hypermutation and a somatic gene conversion-like mechanism in gut-associated lymphoid tissue (GALT).

Even if we did not observed an increase of the B cells population, the oregano (alone or in combination with rosemary) extract orally administered to rabbits probably was able to influence this process of B-cells proliferation in GALT determining a higher responsiveness of these lymphocytes in the *in vitro* proliferation test, in accordance with other reports on chickens.[43]

These findings have been corroborated by the higher production of IgM in the supernatants of the cell cultures derived from the O, OR and E groups (both in presence or absence of mitogens).

Conclusion

Various aromatic plants and their products have been reported to have health and beneficial properties. Data of the present study show that a dietary supplementation with oregano (*Origanum vulgare L.*) may be able to modulate the *in vitro* immune response in rabbits.

Indeed, rabbits receiving oregano exhibited the higher *in vitro* B^+ cell responsiveness together with their higher capacity to produce IgM. These findings recognize the oregano as an aromatic plant with stimulant properties on the adaptive immune responses in rabbits.

However, further research is required to define the optimal dose and/or the combination of these aromatic plant extracts (oregano and rosemary) to improve their immune modulatory effects and to elucidate the mechanisms mediating these actions.

Ethical standards

Animals were handled according to the principles for the care of animals in experimentation (Directive 2010/63/EU).

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Conflicts of Interest

None.

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