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ORIGINAL CONTRIBUTION

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Increased immune response in mice consuming rice bran oil

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Summary *Background* Polyunsaturated fatty acids play a key role in a number of biological functions. Rice bran oil (RBO) is rich in linoleic acid, an essential n-6 fatty acid. n-6 fatty acids are said to have proinflammatory effects as a result of an increase in n-6 fatty acid-derived eicosanoids. RBO is also rich in γ-oryzanol, a compound from the unsaponifiable fraction, with antioxidant properties. *Objective* The aim of this work is to examine the effect of RBO – and/or γ-oryzanol – enriched diets on the regulation of the immune response. *Methods* 4 week-old Balb/C mice were fed diets enriched with either RBO or high oleic-sunflower oil (HOSO), for one month. Serum samples, bone marrow-derived macrophages and lymphocytes from the spleen were collected. *Results* Compared to HOSO, our results show that RBO modulates the immune system by enhancing B-lymphocyte proliferation (6842 ± 2959 vs 10073 ± 4186 cpm; HOSO vs RBO; n = 10 per group) and T_H1-type cytokines such as IL-2 (55.85 ± 18.2 vs 101.7 ± 21.6 pg/ml) or TNF-α (49.12 ± 18.6 vs 184.9 ± 46.2 pg/ml; HOSO vs RBO) in a significant way (n = 10 per group). Moreover, the reduction found in the T_H2 cytokine IL-4 (7.59 ± 2.3 vs 4.48 ± 1.6 pg/ml) and IgE (56.9 ± 39.2 vs 42.4 ± 35.2 ng/ml; HOSO vs RBO, n = 10 per group) levels suggests RBO may have antiallergenic properties. To elucidate the role of γ-oryzanol, a similar study was also carried out including diets enriched with refined RBO or HOSO containing γ-oryzanol (2 %). Our results suggest that although γ-oryzanol may modulate the immune system, it is not responsible for the overall immunostimulation effect seen for RBO. *Conclusions* RBO-enriched diets could be useful in situations where a potentiation of the immune response was required. The fatty acids composition, more than the unsaponifiable fraction, might be responsible for this effect.

Key words inflammation – fatty acids – cytokines – eicosanoids – functional foods

Introduction

There are many factors that can influence the immune system development, maintenance and optimal functioning [1]. Nutrition and nutritional status can have deep effects on immune functions, resistance to infection and autoimmunity [2]. In this sense, certain nutrients can regulate the immune function and both deficiency and excess of them could adversely affect the number and activity of the immune cells. Among nutrients, lipids have a pivotal role in the immune system [3,

4]. They exert different kind of actions which depend on the source, the length and number and position of double bounds of the fatty acids [3, 5].

Much of the interest in lipids and immunity has focused on polyunsaturated fatty acids (PUFA). The presence of PUFA on membrane phospholipids can influence cellular activities because they are metabolized to a variety of lipid mediators, known as eicosanoids, which play important roles in the regulation of the immune and inflammatory responses [6]. Fatty acids also alter membrane fluidity and consequently modulating changes in conformation and/or function of receptors, transporters and enzymes. Moreover, fatty acids also modulate signaling pathways such as MAPK and NF-kB or nuclear receptors such as PPAR and LXR modulating thus gene expression [6].

Rice bran oil (RBO) is an unconventional vegetable oil which in some populations is believed to be healthy [7]. Because of this reason, its consumption is growing, above all in Asian countries. RBO's main components are unsaturated fatty acids, triterpene alcohols, phytosterols, tocotrienols and α-tocopherol. Oleic and linoleic acids constitute the major components of the saponifiable fraction, and the principal component of the unsaponifiable fraction is γ-oryzanol [8, 9].

The antioxidant components, more than its fatty acids, is said to be responsible for RBO's beneficial effects. From a number of studies carried out either in vitro or in vivo, it has been concluded that γ-oryzanol, present in RBO, exerts a hypolipidemic action. Not only does it exert a cholesterol-lowering action but it is also associated with a significant reduction in aortic fatty streak formation [10]. Modulation of pituitary and gastric secretion, involvement in platelet aggregation and in thyroid stimulating hormone release are some other potential properties related to RBO, which suggest other possible therapeutical applications [11, 12].

In spite of the described effects for RBO, no studies about effects on immune system have been carried out. This is the reason why we decided to evaluate the effects of this oil on immune function in healthy animals. We have compared the immunological effects of a RBO enriched diet characterized by a high content of linoleic acid and by the presence of γ-oryzanol, to a high oleic sunflower oil (HOSO)-enriched diet, rich in oleic acid and where there is no γ-oryzanol present. Our results suggest an immunomodulatory action of RBO and its fatty acids seems to be responsible for such an effect. The involvement of γ-oryzanol in this immune response is also clarified. Possible applications of RBO in human nutrition to improve health are suggested.

Material and methods

Reagents and antibodies

Concanavalin A (Con A) and lypopolysaccharide (LPS) were both purchased from Sigma Chemical Co. (St Louis, MO). Antibody kits for ELISAs were purchased from Bethyl (Montgomery, TX) in the case of immunoglobulins or from Biosource (Camarillo, CA) for cytokines. Analysis of histamine (IBL Laboratories) and PGE_2 (Oxford Biomedical Research) levels in different samples were also determined by using other enzyme immunoassay kits.

Animals and diets

Male Balb/C mice (n = 10 per group and experiment) of four weeks of age were purchased from the Granada University breeding colony and housed in a temperature (22 °C) and light-controlled (12 hour) cycle. Guidelines for the care and use of animals were followed as described [13] and experiments were performed in compliance with the Helsinki Declaration and approved by the ethical committee of the Granada University.

Semi-synthetic diets were prepared following the recommendations given by the American Academy of Veterinary for Animal Nutrition. Differences among the diets were only the source of fat included which was 5 % of the diet in each case. Diet compositions are described in Table 1.

Two groups of experiments were performed. In the first group of experiments to analyze the effect of RBO on the immune system, animals were divided into two groups (n = 10 mice per group and experiment) and were fed *ad libitum* either an RBO-enriched diet or an HOSO-enriched diet. This kind of experiment was re-

Table 1 Diet composition

	HOSO	RBO	Refined RBO	HOSO + γ-oryzanol
Energy	4.2	4.2	4.2	4.2
Protein[a]	195	195	195	195
Fat	50[b]	50[c]	50[d]	49[e]
Carbohydrates[f]	629	629	629	629
Fiber[g]	50	50	50	50
Mix vitamin-minerals[h]	10	10	10	10

The units used to express the diet composition are g/kg diet in all cases except for energy which is expressed as Kcal/g diet. [a] Protein was administered as casein (Nutrilac, Arla Foods Ingredients) supplemented with L-methionine (Squim SA). The source of fat was: [b] high oleic sunflower oil (HOSO) (Vandermoortele Iberica), [c] rice bran oil (RBO) (Itochu Corporation), [d] refined RBO (Itochu Corporation), [e] 49 g/kg of HOSO supplemented with 1 g/kg of γ-oryzanol (Tokio Kasei). [f] Carbohydrates were administered as starch corn (Cerestar S. A.) plus 50 g/kg of sucrose (Ebro-Puleva S. L.). [g] Fiber was included as α-cellulose (Sigma). [h] The requirements of mineral and vitamins were administered by a mixture purchased from Nuriiespadales SA

peated three times with similar results. The second group of experiments, dedicated to elucidate the components in RBO involved in the effects of RBO on the immune system, consisted of four groups of animals (also n = 10 per group and experiment): HOSO, RBO, refined RBO, while the last group was fed a γ-oryzanol-enriched diet (2% of the fat) using HOSO as the source of fat. Crude RBO contains 1.9% of γ-oryzanol, while HOSO and refined RBO were absent of the antioxidant. In the refined RBO the unsaponifiable fraction was completely eliminated. During the studies a control of the behavior and physical aspects was carried out by periodical observations. In this case, the experiment was conducted independently two times. In all cases, after four weeks, the animals were sacrificed by intraperitoneal administration of sodium penthotal (50 mg/kg body weight) and blood was collected by cardiac puncture into EDTA tubes. Blood was then centrifugated 2200 x g 10 min at 4 °C and plasma aliquots were collected and frozen at –80 °C until needed.

Cell culture and proliferation assay

Lymphocytes from spleen and bone marrow-derived macrophages were obtained and cultured as previously described [14, 15]. Spleen-derived lymphocytes were cultured in 24-well plates (2×10^6 cells/well) in 1 ml of medium and stimulated with ConA (5 μg/ml) or LPS (50 μg/ml). [^{3}H]-thymidine (1 μCi/ml) (Amersham Biosciences, Arlington Heights, IL) incorporation was measured as previously described [14]. Radioactivity was counted by liquid scintillation using a 2100 Tri-Carb Packard (Meriden, CT) scintillation counter. Each point was performed in duplicate.

Analysis of cytokine production

Spleen-derived lymphocytes were cultured in 6-well plates (10×10^6 cells/well) in 5 ml of medium and incubated for 24 h in the presence or absence of ConA (5μg/ml) or LPS (50 μg/ml). The supernatants were collected after 48 h of incubation. Differentiated macrophages were cultured in 6-well plates (3×10^6 cells/well). After 12 hours, adherent macrophages were stimulated with LPS (100 ng/ml). Supernatants were collected after 24 hours. Concentrations of IgA, IgG1, IgE, IL-2, IL-1β, IL-4, TNF-α, and IFN-γ were determined by ELISA according to the manufacturer's instructions (Bethyl or Biosource) in supernatants from lymphocytes and for macrophages cultures as indicated. Serum samples were also analyzed. The samples from all the mice were analyzed.

Analysis of gene expression by RT-PCR

Total mRNA was isolated from cultured spleen cells by using Trizol Reagent (Invitrogen, Carlsbad, CA) as described by the manufacturer. The reversed transcription was performed by using the Reverse Transcriptase AMV (Roche, Indianapolis, IN) as described elsewhere. First-strand cDNAs were either stored at –20 °C or used for the PCR step. PCR reactions (25 μl total volume) were carried out as previously reported [16]. Murine oligonucleotides primers used in the PCR were purchased from MWG-Biotech AG (Ebersberg, Germany) and the nucleotide sequences were as follows: IFN-γ, for: 5'-TGGAGGAACTGGCAAAAGGATGGT-3', rev: 5'-TTGGCACAATCTCTTCAC-3'; IL2, for: 5'-TGATGGACCTACAGGAGCTCCTGA-3', rev: 5'-GAGTCAAATCCACAACATGCCGCA-3'; IL-1β, for: 5'-TGATGAG-AATGACCTGTTCT-3', rev: 5'-CTTCTTCAAAGATGAAGGAAA-3'; IL-4, for: 5'-ACGAGGTCACAGGAGAAGGGAC-3', rev: 5'-GGAGCAGCTTATCGATGAATCC-3'; TNF-α, for: 5'-AACTAGTGGTGCCAGCCGAT-3', rev: 5'-CTTCACAGAGCAATGACTCC-3'; β-actin, for: 5'-TGGAATCCTGTGGCATCC-3', rev: 5'-AACGCAGCTCAGTAACAGTCC-3'. Each RT-PCR was performed with five of the samples from each group obtaining similar results.

Statistical analysis

In all cases, differences between means were tested for statistical significance using a one-way analysis of variance (ANOVA) and *post hoc* least significance tests. The data in the text and figures are always represented as the mean ± S. D.

Results

Diets, mice growth and behavior

The amount of the diets consumed was determined daily by weighing the total diet ingested per day, and no significant differences were observed between groups during the study (data not shown). Taking all data together, each mouse consumed approximately 6.18 ± 0.77 g/day of the diets. We calculated, from the analysis of fatty acids content in the diets, the daily intake of linoleic and oleic fatty acids, the main fatty acids components of both diets (Table 2). In this sense, animals fed with the HOSO diet consumed approximately 40 ± 0.5 and 235 ± 4.5 mg of linoleic and oleic acid per day, while animals receiving the RBO diet consumed 110 ± 1.5 and 130 ± 2.5 mg per day. Therefore, RBO supplied triple the amount of linoleic acid and half the amount of oleic acid compared to HOSO diets. Consequently, RBO diet could

Table 2 Fatty acid composition of the diets

	HOSO	RBO	Refined RBO
C10:0	0.11	0.07	0.14
C12:0	0.05	nd	nd
C14:0	0.07	0.43	0.41
C16:0	5.14	16.27	15.94
C16:1	0.12	0.21	0.2
C17:0	0.04	nd	0.04
C18:0	4.04	1.84	1.86
C18:1n9c	75.14	41.92	42.3
C18:1n7	nd	0.89	0.85
C18:2n6tt	0.51	nd	0.79
C18:2n6c	12.98	35.44	34.72
C18:3n6 (gamma)	0.05	nd	0.24
C18:3n3 (alpha)	0.15	1.24	0.62
C18:3n4	0.05	nd	0.21
C20:1n9	0.23	0.49	0.51
C21:0	0.04	nd	0.08
C22:0	0.75	0.17	0.16
C24:0	0.2	0.36	0.21
C22:5n6	nd	0.05	nd

nd Not detected; results expressed as percentage

be considered rich in n-6 polyunsaturated fatty acids (35.44 % vs 12.98 %; RBO vs HOSO diets), while a HOSO diet was rich in monounsaturated fatty acids (75.11 % vs 42.3 %; HOSO vs RBO diets).

Although there were differences in the fatty acid composition of the diets, the final body weight and the total growth rate of the animals did not differ between the mice fed on the different diets (data not shown). Moreover, no side effects such as occurrence of diarrhea or differences in either the physical aspects nor the behavior were observed in the mice from the different groups.

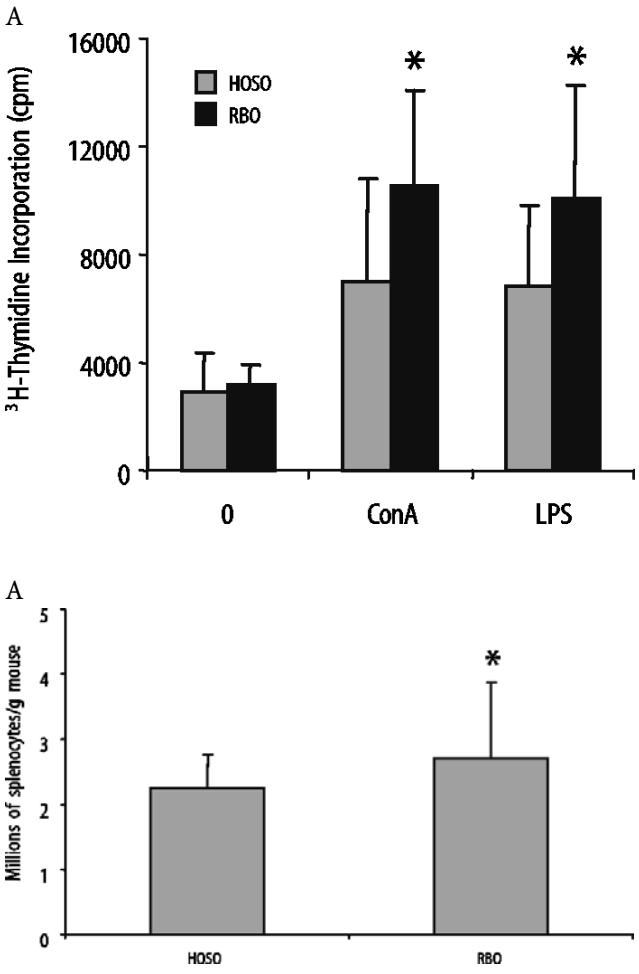

Fig. 1 RBO diet enhances lymphocyte proliferation: **A** T and B lymphocyte proliferation measured by ^{3}H-thymidine incorporation. Proliferation levels (expressed in cpm) of non-stimulated [0] and Con A and LPS stimulated splenocytes from mice consuming HOSO or RBO diets. **B** Millions of splenocytes per g of mouse consuming HOSO or RBO diets measured using an hemocytometer. Values are mean ± SD, n = 10. * Significant difference between HOSO and RBO group ($P < 0.05$)

RBO enhances mainly T_H1 lymphocyte activities

Both lymphocytes T and B from RBO-fed mice significantly increased their proliferation ratio (Fig. 1A) in comparison with HOSO-fed animals. This higher proliferation ratio was also reflected as an increase in the number of splenocytes per mice (Fig. 1B).

We explored the association between proliferation and activation of lymphocytes by testing if the RBO diet would modify the expression of cytokines by these cells. In this sense and in agreement with the increased lymphocyte proliferation, we observed a higher production of IL-2 by lymphocytes in RBO-fed mice (Fig. 2A). In addition, other T_H1-type cytokines were also increased in the RBO group, including IFN-γ and TNF-α (Fig. 2), which corresponded with an increase of the gene transcription as analyzed by RT-PCR (Fig. 2). Finally, we also observed a trend to increase the expression of other T_H1 cytokines such as IL-12 (199.1 ± 8.4 vs 266.9 ± 12.9 pg/ml; HOSO vs RBO) in RBO-fed mice when compared with the HOSO group.

T_H2 responses were lower in RBO group. This is the case, for example, of the levels of IL-4 in serum or in the supernatants obtained from the cultured spleen-derived lymphocytes (Fig. 3A, B) or in the lymphocyte-derived IL-5 expression (30.3 ± 6.2 vs 10.5 ± 2.1 pg/ml; HOSO vs RBO). Due to the reduction in T_H2 cytokines, we have also observed that RBO was able to reduce the amount of both allergy-related inmunoglobulins in mice, namely IgE (Fig. 3C) and IgG1 (75.18 ± 39.87 vs 50.43 ± 24.18 µg/ml; HOSO vs RBO). Finally, we also stated a reduction in the plasma levels of histamine (10.32 ± 2.2ng/ml vs 3.11 ± 0.92; HOSO vs RBO) although they did not reach statistical significance.

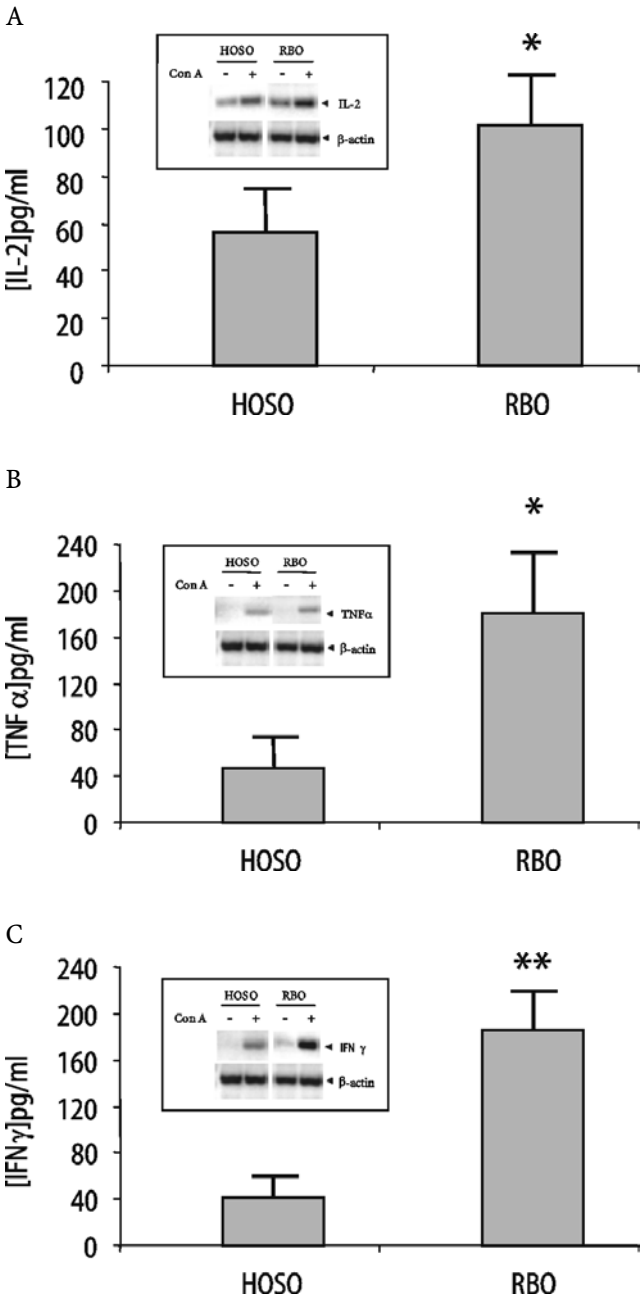

Fig. 2 Increase in T_H1 lymphocyte response by RBO: Levels of IL-2 (**A**), TNF-α (**B**) and IFN-γ (**C**) measured by ELISA in supernatants of cultured stimulated splenocytes from mice consuming HOSO or RBO diets. The stimulus used was Con A (5 μg/ml). Con A was used as stimulus. Results are expressed in pg/ml as mean ± SD. * Significant difference between HOSO and RBO group ($P < 0.05$) or ** ($P < 0.01$). Gene expression of the cytokines in the previous splenocytes was analyzed by RT-PCR using β-actin as a control and showed in the frame inside the figure

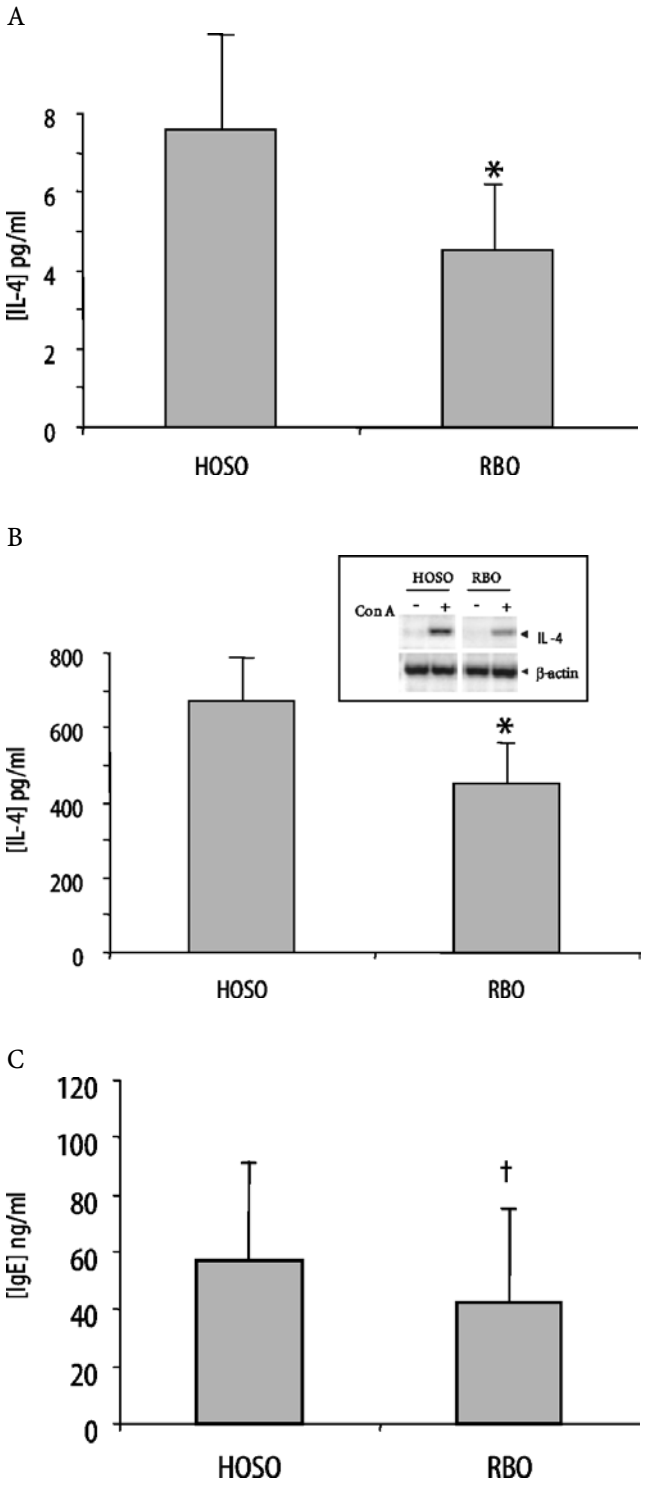

Fig. 3 Decrease of T_H2 response by RBO: **A, B** Levels of IL-4 in serum and in supernatants from stimulated lymphocytes measured by ELISA and expressed as pg/ml. Gene expression in such splenocytes were measured by RT-PCR, using β-actin as a control. **C** IgE serum levels analyzed by ELISA from mice consuming HOSO or RBO expressed as ng/ml. Results are mean ± SD. * Significant difference between HOSO and RBO group ($P < 0.05$). † $P = 0.06$

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RBO also enhances macrophage responses

We have observed that immune response of primary macrophages from the RBO group were also higher compared to the HOSO group. Macrophages from RBO-fed mice generated a greater amount of TNF-α or IL-1β (Fig. 4) when stimulated ex vivo with LPS as compared with those fed the HOSO diet. Again, these results were also in concordance with an increase in the related gene expression (Fig. 4).

RBO diets did not modify the macrophage proliferation rate induced by M-CSF (data not shown), in contrast to what we observed in lymphocytes. This could be due to the fact that macrophage activation is not related to an induction of the proliferation of these types of cells as opposed to lymphocytes [14, 17].

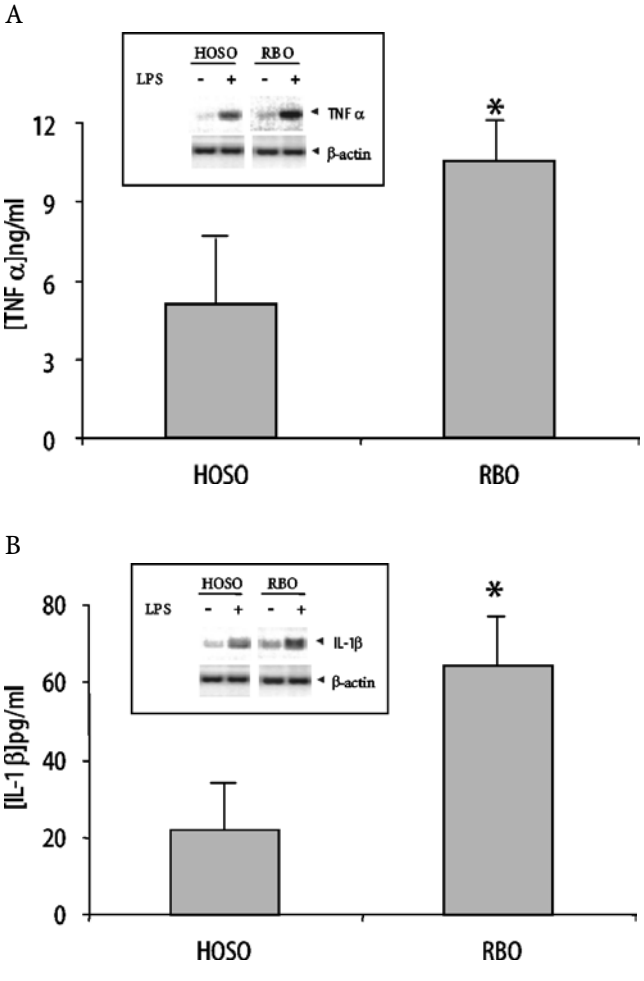

Fig. 4 Increase in T_H1 bone marrow derived-macrophage response by RBO: Production of TNF-α (**A**) and IL-1β (**B**) by LPS stimulated bone marrow derived-macrophages measured by ELISA and expressed as mean ± SD. Gene expression was analyzed by RTPCR using β-actin as a control. * Significant difference between HOSO and RBO ($P < 0.05$)

Fatty acids but not γ-oryzanol are responsible for most of the immunostimulatory effects of RBO

To better understand whether the RBO effects were due to the fatty acids composition or to the γ-oryzanol content, a second group of experiments was performed including diets with refined RBO and enriched with γ-oryzanol as described in the material and methods section. The results observed with the RBO and HOSO diets were identical to those observed previously. Surprisingly, our results showed that the fatty acid composition of the RBO diet but not the unsaponifiable fraction was the main responsible for the immunomodulation observed with these types of RBO-based diets. In this sense, we observed the same effects, namely an increase in T_H1 response and a reduction in the T_H2 response, in the mice treated with both RBO and refined RBO diets, measured as lymphocyte proliferation, TNF-α or IL-4 expression (Fig. 5). However, although the HOSO+γ-oryzanol diet was able to also increase TNF-α expression, it had no effect on lymphocyte proliferation or T_H2 response as measured by IL-4 expression (Fig. 5), suggesting that the unsaponifiable fraction of RBO was not responsible for most of the immunoregulatory effects produced by the RBO diet. Moreover, the implication of the fatty acid fraction was also suggested by the fact that plasma from RBO-fed mice had higher concentrations of PGE_2 (7.57 ± 1.81 vs 9.85 ± 1.32 ng/ml; HOSO vs RBO).

Discussion

A balanced and adequate nutrition is required for the development, maintenance and optimal functioning of the immune system [3]. Nutrient-related factors that have the most pronounced effects on the immune system are fatty acids, protein-energy malnutrition and certain vitamins and minerals [3, 18, 19]. Several parameters of the immune system are said to be modified by dietary lipid administration.

The saponifiable RBO fraction is mainly composed of n-6 fatty acids, while a compound with antioxidant properties, γ-oryzanol, constitutes the unsaponifiable fraction [20]. There are a number of studies with RBO suggesting cardiovascular beneficial effects, normally attributed to γ-oryzanol [21, 22]. Animal and human studies have shown how RBO may lower serum cholesterol levels, inhibit platelet aggregation, and some other risk factors for cardiovascular disease.

However, to the best of our knowledge, no studies on the effects of RBO in the immune system have been carried out. Nevertheless, other studies using diets with similar fatty acid contents have been developed to check the immunological effects of oleic and linoleic acids [23]. Our results suggest immunomodulatory effects pro-

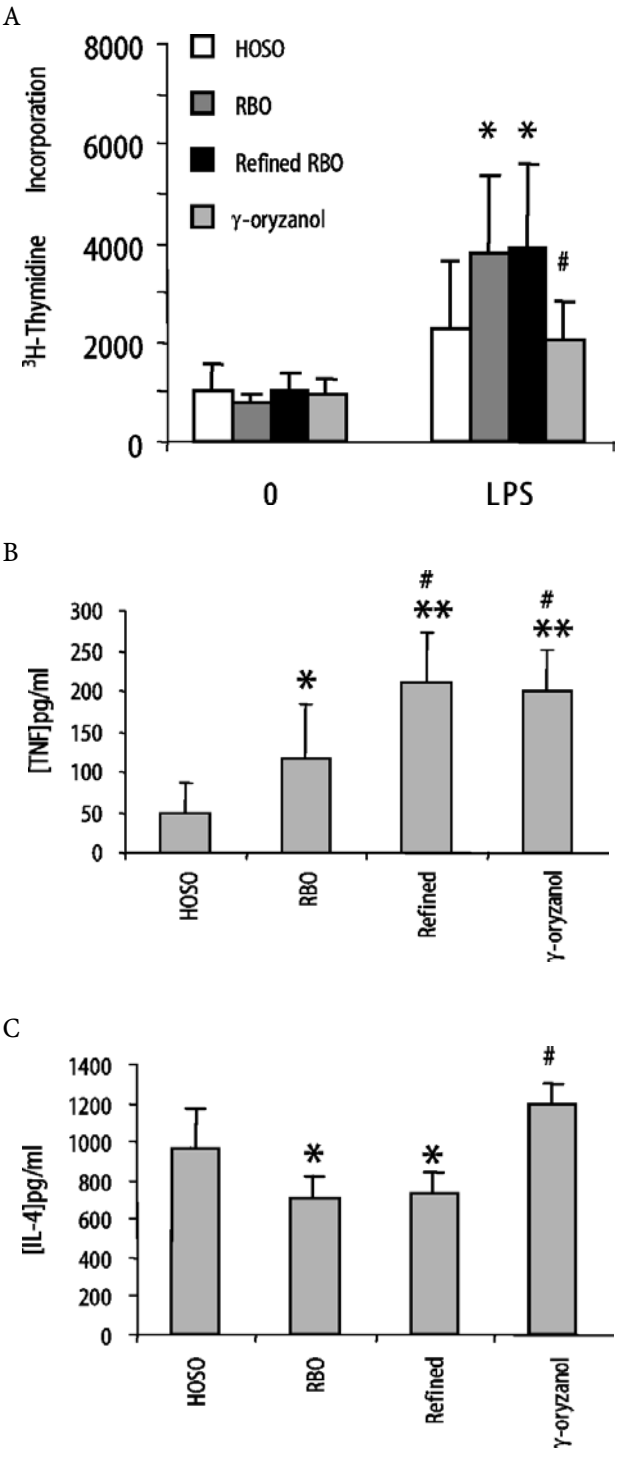

Fig. 5 γ-oryzanol is not responsible for the immunostimulation attributed to RBO: **A** B-lymphocyte proliferation as measured by ^{3}H-thymidine incorporation in basal and stimulated splenocyte cultures. Results expressed in cpm. **B, C** Levels of TNF-α and IL-4 in lymphocyte supernatants stimulated with Con A, measured by ELISA and expressed in pg/ml. The culture of the splenocytes studied derived from mice consuming HOSO, RBO, refined RBO or enriched γ-oryzanol diet. Results are expressed as mean ± SD. * Significant difference between HOSO and rBO group ($P < 0.05$) or ** ($P < 0.01$). # Significant difference between RBO and γ-oryzanol groups ($P < 0.05$)

duced by the consumption of RBO, which also indicate that γ-oryzanol is not responsible for the overall immunoregulation despite having important roles in other organism functions. In fact, γ-oryzanol modifies the immune response in a different way to the one observed for RBO. Thus, fatty acid composition might be responsible for the effects of RBO. A high linoleic acid consumption could be responsible for the increase in lymphocyte proliferation, spleen size and T_H1 response as suggested by other in vivo and in vitro studies using linoleic acid [24–26]. RBO contains an important fraction of linoleic acid, and the effects described in the above mentioned studies are also observed in our study. This immunomodulatory effect is in part due to the increased lymphocyte proliferation which leads to increased synthesis of the T_H1 cytokines responsible for the defence of the organism. Since γ-oryzanol is unable to enhance lymphocyte proliferation, we speculate that γ-oryzanol is not responsible for the large amount of cytokines produced.

As a result of the higher T_H1 response, the T_H2 response may also be modified. Due to the duality in the equilibrium of T_H1 and T_H2 cytokines in the regulation of the immune system (in concordance with the tendency through a T_H1 bias induced by the RBO-enriched diets), T_H2 response is lower in the RBO group. Because of this, RBO may have not only indirect antibacterial properties but also antiallergenic ones. As people who suffer from atopic dermatitis are more susceptible to infections [27], RBO could ameliorate both allergic and infective processes in these cases. However, further studies must be carried out to obtain more conclusive data.

The reduced T_H2 response is not an effect produced by γ-oryzanol. As a consequence, neither the higher T_H1 response nor the T_H2 reduction produced by RBO consumption seem to be modulated by the antioxidant fraction. In spite of this, other advantages attributed to γ-oryzanol or RBO could make this oil an important alternative to reach nutritional targets for health. However, due to the exacerbated response of the immune system observed with RBO, the generalized use of this oil, not γ-oryzanol, as a cardiovascular protector has to be treated with caution since atherosclerosis has been suggested to be an inflammatory disease [28] that could be impaired.

In conclusion, the inclusion of one or another sort of fat in the diet could bring important consequences for the immune system as we have shown in this study. The way in which the different kinds of fat regulate the immune system must be taken into account to choose the proper diet for each subject. In order to improve human well-being, RBO could be included in the diet to obtain a fortification of organism defences. But not only improving normal health status would be a reason for its consumption, but also an alternative for certain physiological conditions where a stimulation of the immune system is required. This is the case in the elderly, whose immune function may be altered by age [29], or during childhood

where susceptibility to infections may be increased due to frequent exposure to pathogens or insufficient development of their immune system. During strong physical activity, organism defences are also altered [30] and RBO could help with the restoration of the normal immune parameters. However, immunomodulation which takes place after RBO consumption could be a drawback for people suffering from autoimmune or inflammatory diseases because it favors an inflammatory response which could impair their illnesses as previously related to the case of atherosclerosis.

RBO modulates the immune function in different ways and depending on the circumstances, its consumption may or may not be recommended. Results from this study do not provide enough information to make recommendations with respect to this. More studies are necessary in order to elucidate the effectiveness of RBO in immune depressing conditions or its detrimental effects on inflammatory diseases.

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