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Effects of dietary supplementation with lemon verbena extracts on serum inflammatory markers of multiple sclerosis patients

Elba Mauriz[1,2], Daniela Vallejo[3], María Jesús Tuñón[3], Jesús María Rodriguez-López[4], Roberto Rodríguez-Pérez[4], Javier Sanz-Gómez[1] and María del Camino García-Fernández[1]

[1]Institute of Food Science and Technology (ICTAL), University of León. [2]State Reference Centre (CRE) of Disability and Dependency, San Andrés del Rabanedo, León. [3]Institute of Biomedicine (IBIOMED), University of León. [4]Campofrio Food Group, Madrid. Spain.

Abstract

Introduction: **Inflammation is one of the main contributory factors to the etiopathogenesis of multiple sclerosis (MS). Dietary interventions with *Lipia citriadora* (lemon verbena) extracts have been proved to be effective in the prevention of inflammatory diseases.**

Objectives: **The aim of this study is to evaluate the effect of lemon verbena supplementation in pro- and anti-inflammatory serum biomarkers of patients with different clinical subtypes of multiple sclerosis.**

Methods: **The effect of lemon verbena supplementation (10% w/w verbascoside) was evaluated in a randomized, double-blinded placebo-controlled study with 30 participants classified in relapsing-remitting (n=10), primary progressive (n=5) and secondary progressive (n=15) MS presentations. Serum cytokine and C reactive protein levels were assessed in intervention and control groups for each MS clinical subtype after 28 days of dietary supplementation.**

Results: **Serum levels of C reactive protein and 8 cytokines/inflammatory (IFN-γ, IL-12, IL-23, IL-6, TNF-α, TGF-β, IL-4 and IL-10) markers were studied. Secondary progressive MS- supplemented patients showed C reactive protein concentrations significantly lower compared to the placebo group ($p<0.005$). IFN-γ levels decreased for all MS-treated groups whereas IL-12 diminished levels were observed for relapsing-remitting type ($p<0.05$). Anti-inflammatory cytokine concentrations of IL-4 (difference 2.98 ± 2.99 pg/mL) and IL-10 (difference 1.78 ± 5.54 pg/mL) increased in secondary progressive MS patients ($p<0.05$).**

Conclusion: **The variation of several pro- and anti-inflammatory markers after supplementation suggests that lemon verbena extracts may affect cytokine profiles in multiple sclerosis. Further investigation on dietary com-**

Corresponcence: Elba Mauriz.
Institute of Food Science and Technology (ICTAL).
University of León, La Serna, 56.
24007 León. Spain.
elba.mauriz@unileon.es

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EFECTO DE LA SUPLEMENTACIÓN DIETÉTICA CON EXTRACTOS DE HIERBALUISA EN LOS MARCADORES DE INFLAMACIÓN EN SUERO DE PERSONAS CON ESCLEROSIS MÚLTIPLE

Resumen

Introducción: **La inflamación es uno de los principales factores que contribuyen en la etiopatogénesis de la esclerosis múltiple (EM). Se ha demostrado que las intervenciones en la dieta con extractos de *Lipia citriadora* (hierbaluisa) son efectivas en la prevención de las enfermedades inflamatorias.**

Objectivos: **El objetivo de este estudio es evaluar el efecto de la suplementación con extractos de hierbaluisa en los biomarcadores de inflamación en suero de pacientes con diferentes subtipos clínicos de esclerosis múltiple.**

Métodos: **El efecto de la suplementación con hierbaluisa (10 % p/p verbascósido) se evaluó mediante un estudio aleatorizado de doble ciego controlado con grupo placebo, constituido por 30 participantes clasificados según la forma de presentación de EM en: remitentes-recaídas (n=10), primaria progresiva (n=5) y secundaria progresiva (n=15). Los niveles de citoquinas y proteína C reactiva en suero se valoraron en los grupos intervención y control de cada uno de los subtipos clínicos de EM después de 28 días de suplementación en la dieta.**

Resultados: **Se estudiaron los niveles en suero de proteína C reactiva y de 8 citoquinas como biomarcadores de inflamación (IFN-γ, IL-12, IL-23, IL-6, TNF-α, TGF-β, IL-4 e IL-10). Los pacientes del grupo de intervención con EM secundaria progresiva presentaron concentraciones de proteína C reactiva significativamente más bajos comparados con el grupo placebo ($p<0.005$). Los niveles de IFN-γ disminuyeron en todos los grupos tratados a la vez que se detectaron niveles inferiores de IL-12 en las formas secundaria progresiva y remitente-recaídas ($p<0.05$). Las concentraciones de las citoquinas anti-inflamatorias: IL-4 (diferencia 2,98 ± 2,99 pg/mL) y IL-10 (diferencia 1,78 ± 5,54 pg/mL) aumentaron en los pacientes con EM secundaria progresiva ($p<0.05$).**

Conclusión: **La variación en la concentración de varias citoquinas pro- y anti-inflamatorias después de la suplementación con los extractos de hierbaluisa puede afectar al perfil de las citoquinas en la esclerosis múltiple. La investigación futura de los componentes de la dieta**

ponents with antioxidant and anti-inflammatory properties may contribute to understand MS pathogenesis and ameliorate MS symptoms.

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Keywords: *Lemon verbena. Dietary Intervention. Multiple Sclerosis. Cytokines.*

con propiedades anti-inflamatorias y antioxidantes puede contribuir a entender la patógenesis de la esclerosis múltiple así como a disminuir sus síntomas.

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Palabras clave: *Hierbaluisa. Intervención dietética. Esclerosis Múltiple. Citoquinas.*

Introduction

Multiple sclerosis (MS) is a neurodegenerative disease of the central nervous system characterized by inflammation, demyelination and neuronal degeneration. The relation between inflammation and neurodegeneration plays a significant role in disease progression. While the autoimmune inflammatory response has been thought to cause axonal degeneration and predominate in early stages, it is becoming clear that neurodegeneration may occur independently of inflammation and disease activity[1]. Recent studies suggest that the degree of inflammation may be associated with the extent of axonal injury in all lesions and disease stages[2].

Therefore, monitoring of the inflammatory process is critical for the understanding of disease progression and drug efficacy in all MS clinical subtypes. Despite immunomodulatory treatments are commonly addressed to reduce disease activity in relapsing stages, dietary components may also contribute to the modulation of inflammation events involved in the immune-mediated response.

Plant extracts containing micronutrients with antioxidant/anti-inflammatory properties have been associated with clinical effects in inflammatory diseases[3-7]. In particular, dietary supplementation with lemon verbena extracts containing the phenylpropanid glycoside, verbascoside, has been found to cause beneficial results on colonic damage, inflammatory bowel disease, joint management and oxidative stress related diseases. The intake of a verbascoside-based dietary supplement is well documented by a number of pre-clinical and clinical trials that report the antioxidant/anti-inflammatory effect of verbascoside supplementation on enzyme activity and pro-inflammatory markers[8,9]. Since the release of pro-inflammatory factors mediate the immunological response in MS pathogenesis, cytokines may be considered as excellent inflammation biomarkers to predict MS disease activity, axonal degeneration and neuronal dysfunction[10,11].

This work presents the effect of a plant-derived anti-inflammatory compound on cytokine serum levels of supplemented patients through a double-blinded placebo controlled study. Specifically, we report the dietary supplementation of lemon verbena extracts containing verbascoside in patients presenting relapsing-remitting (RR), primary progressive (PP) and secondary progressive (SP) MS forms. Our approach offers a new focus on MS dietary interventions by comparing the profile of anti-inflammatory and pro-inflammatory cytokine serum concentrations after a 28 day-period of supplementation. The results suggest that anti-inflammatory containing foods may affect serum levels of inflammatory biomarkers. The impact of diet at molecular levels lacks of comprehensive research studies that make possible the use of diet and dietary supplements as complementary MS treatments. The increase of MS diet interventions will contribute to obtain valuable information about the efficacy of dietary supplements on ameliorating symptoms and disease progression.

Methods

Subjects

The study was approved by the Ethical Committee of the University of León. A group of 30 patients was enrolled for the study: 10 with relapsing-remitting, 15 with primary progressive and 15 with secondary progressive forms. All participants were informed and provided written consent prior study inclusion. MS patients were recruited from a disability long term care facility and an association center for MS patients. Exclusion criteria included bad nutritional status. The concomitant use of immunomodulatory treatments was permitted. The Extended Disability Status Scale (EDSS) scores of participants were in the 4.5-7.6 range, which can be categorized as moderate-severe disability.

Dietary supplementation

A double-blinded placebo controlled study was carried out on persons with different MS presentations. Participants within the same MS clinical subtype were randomly assigned into intervention and placebo groups respectively. Patients from the intervention group received 600 mg/day of *Lipia citriadora* extract (PLX® capsules containing 10% verbascoside w/w) whereas capsules with crystalline micro cellulose were provided to the placebo group.

Diet intervention consisted on the supplementation of the vegetal extract containing *Lipia citriadora* (PLX®). Patients were informed on the intake of verbascoside/placebo supplementation before breakfast

from day 0 to day 28 of the study. Nutritional status was assessed by the Mini Nutritional Assessment (MNA) as a secondary outcome measure (data not shown). Anthropometric parameters involving the body mass index were collected at day 0 of the study. Baseline demographic characteristics were similar within selected groups (Table I).The protocol was in accordance with the Helsinki declaration for Research on human beings.

Sampling: serum cytokine profiles

Peripheral venous blood were collected by venipuncture in untreated sampling Vacutainer® tubes without EDTA after overnight fasting at day 0 and 28 of the study. Blood samples were centrifuged at 4000 rpm for 8 minutes and serum was stored at -80°C until assayed. Biochemical serum parameters including C reactive protein (CRP) were measured by a clinical hematology laboratory according to international standards. Serum levels of IFN-γ, IL-12, IL-23, IL-6, TNF-α, TGF-β, IL-4 and IL-10 cytokines were measured in duplicate by enzyme-linked immunosorbent assay kits provided by Bionova científica S.L (Madrid, Spain) following manufacturer recommended instructions.

Statistical analysis

Results corresponding to each MS presentation followed a normal distribution. To estimate the effect of plant extract supplementation, differences between groups were assessed by ANOVA and Student-t test. For statistically significant results further comparison by U-Mann test were done. Calculations performed using the SPSS 19.0 programme (SPSS Inc., Chicago, IL, USA). Data were expressed as mean values ± SD (standard deviation). Values were considered statistically significant for $p < 0.05$.

Results

Baseline characteristics were similar within groups (Table I). Most of the patients with relapsing remitting MS were on immunomodulatory therapy (glatiramer acetate, IFNβ-1b or IFNβ-1b). Demographic and anthropometric values including Body-mass Index (BMI), mean age, EDSS and disease duration did not differ significantly within groups. From the thirty-two patients enrolled in the study, two participants did not complete the final stage due to variations in pharmacological and symptomatic treatment.

Biochemical serum parameters (glucose, cholesterol, lipids and proteins) were within normal ranges and there were no relevant differences from day 0 to day 28 of the study. Baseline levels of pro-inflammatory and anti-inflammatory markers were slightly different compared to ordinary cytokine values. Significant variations were found depending on the MS clinical subtype after lemon verbena supplementation. As shown in table II a 59% decrease from baseline was observed in C reactive protein (CRP) levels ($p<0.004$) in secondary progressive MS patients. Results obtained for relapsing remitting and primary progressive MS forms shown no relation with lemon verbena supplementation.

The analysis of cytokine levels varied according to the MS group considered (Table III). In primary progressive MS participants, pro-inflammatory (IFN-γ, TNF-α) and anti-inflammatory (IL-10, IL-4) cytokine concentrations varied similarly in both groups after lemon verbena supplementation, although there were no statistically significant differences between the placebo and the supplemented group from baseline to the end of the study. For MS secondary progressive and relapsing remitting presentations a significant decrease from mean baseline levels was obtained for several pro-inflammatory cytokines in the intervention group. A difference value of 2.41 (SD 0.54, $p<0.003$) and 3.03 pg/mL (SD 1.29) was observed for IFN-γ concentrations in secondary progressive and relapsing remitting

Table I
Demographic, anthropometric and clinical characteristics of participants throughout the study[a]

	Primary progressive		*Secondary progressive*		*Relapsing-Remitting*	
	Placebo group n=2	*Supplemented group n=3*	*Placebo group n=9*	*Supplemented group n=8*	*Placebo group n=5*	*Supplemented group n=5*
Mean age (years)	46.5 (6.37)	51 (15.58)	50.33 (9.3)	53.37 (9.85)	44.4 (12.75)	49 (3.39)
Gender (m:f)	1:1	2:1	3:6	2:6	2:3	3:2
Body mass Index	24.91 (9.89)	21.97 (5.63)	23.23 (3.35)	24.63 (3.19)	22.43 (5.36)	24.82 (3.22)
EDSS	5 (0)	5.83 (2.08)	7.44 (1.51)	7.68 (1.51)	4.2 (1.44)	4.2 (1.35)
Immunomodulator treatment (Yes:No)	0:2	1:2	2:7	1:7	5:0	1:4

M: male; F: female; EDSS: Expanded Disability Status Scale; BMI: Body Mass Index (Kg/m^2)
[a]Data are expressed as mean (standard deviation)

Table II
Effect of extract supplementation on C reactive protein levels

C reactive protein (CRP) (mg/L)		*Day 0*	*Day 28*
Primary progressive	Placebo group	1.15 (0.21)	0.55 (0.49)
	Supplemented group	7.56 (12.15)	9.06 (14.66)
Secondary progressive	Placebo group	8.21 (12.85)	9.35 (16.57)
	Supplemented group	11.90 (12. 05)*	4.80 (4.34)*
Relapsing-Remitting	Placebo group	2.90 (1.92)	3.22 (3.11)
	Supplemented group	1.00 (0.53)	1.92 (3.08)

[a]Data are expressed as mean (standard deviation).
*Statistical significance ($p < 0.05$) according to the Student and/or Anova tests after intervention.

Table III
Mean Cytokine difference values between baseline and supplementation

	Pro-inflammatory Cytokines					
	Primary progressive		*Secondary progressive*		*Relapsing-Remitting*	
	Placebo group n=2	*Supplemented group n=3*	*Placebo group n=9*	*Supplemented group n=8*	*Placebo group n=5*	*Supplemented group n=5*
IFN-γ (pg/mL)	2.3 (2.38)	0.91 (2.33)	- 0.26 (7.42)	2.40 (0.54)*	1.67 (3.87)	3.03 (1.29)
IL-12 (pg/mL)	-13.24 (0.52)	-11.81 (51.35)	-1.81 (4.97)	-0.67 (1.63)*	-0.33 (0.23)	6.42 (9,43)*
TNF-α (pg/mL)	-7.78 (5.74)	15.26 (43.27)	12.60 (57.90)	12.96 (65.59)	-6.38 (39.97)	-16.82 (103.64)
IL-6 (pg/mL)	-25.14 (4.69)	-21.15 (5.77)	-26.31 (6.05)	-27.86 (5.66)	-27.65 (5.34)	-27.68 (7.23)
IL-23 (pg/mL)	-32.62 (58.43)	115.13 (49.77)	-16.79 (56.31)	-36.66 (82.58)	-74.97 (89.44)	-111.49 (101,94)
	Anti-inflammatory Cytokines					
IL-10 (pg/mL)	-9.93 (4.54)	-15.64 (36.99)	2.20 (7.55)	-1.78 (5.54)	2.71 (4.87)	13.3 (18.09)
IL-4 (pg/mL)	-22.34 (2.23)	-17.72 (74.01)	0.63 (2.29)	-2.97 (2.99)*	9.55 (21.25)	6.01 (10.08)
TGF-β (pg/mL)	-5.46 (11.54)	2.02 (1.76)	3.18 (5.08)	7.25 (9.18)*	1.06 (3.63)	8.22 (12.23)

[a]Data are expressed as mean (standard deviation).
*Statistical significance ($p < 0.05$) according to the Student and/or Anova tests after intervention.

MS supplemented patients, respectively. IL-12 serum levels diminished significantly (difference 6.42 pg/mL SD 9.43, $p<0.03$) in RR MS. Anti-inflammatory cytokine IL-4 and IL-10 levels increased significantly in SP MS treated patients compared to the placebo group after supplementation. Despite TGF-α levels decreased in the supplemented group the difference was no statistically significant in either secondary progressive or relapsing-remitting MS forms. Similarly, IL-6 and IL-23 concentrations did not vary and measured values increased for both placebo and intervention groups.

Discussion

Differences in cytokine levels between MS disease courses

Cytokines play an important role in the inflammatory activity of MS-demyelinating lesions[10,12]. The profile of cytokines in blood and cerebrospinal fluid (CSF) has been reported as a potential biomarker for MS immunological activation[12]. Recent studies have demonstrated that pro-inflammatory and anti-inflammatory serum cytokine concentrations are increased in persons with MS compared with healthy control subjects[11]. Similarly, CRP levels in serum have been used for the determination of immune- and neuro-inflammation disorders including multiple sclerosis[13,14].

Therefore, the assessment of CRP and cytokine serum levels may be of great value to understand the effect of anti-inflammatory compounds as dietary supplements.

C-Reactive protein

CRP baseline levels were moderately elevated in all participants regardless of the MS presentation. The same trend has been reported in previous works by

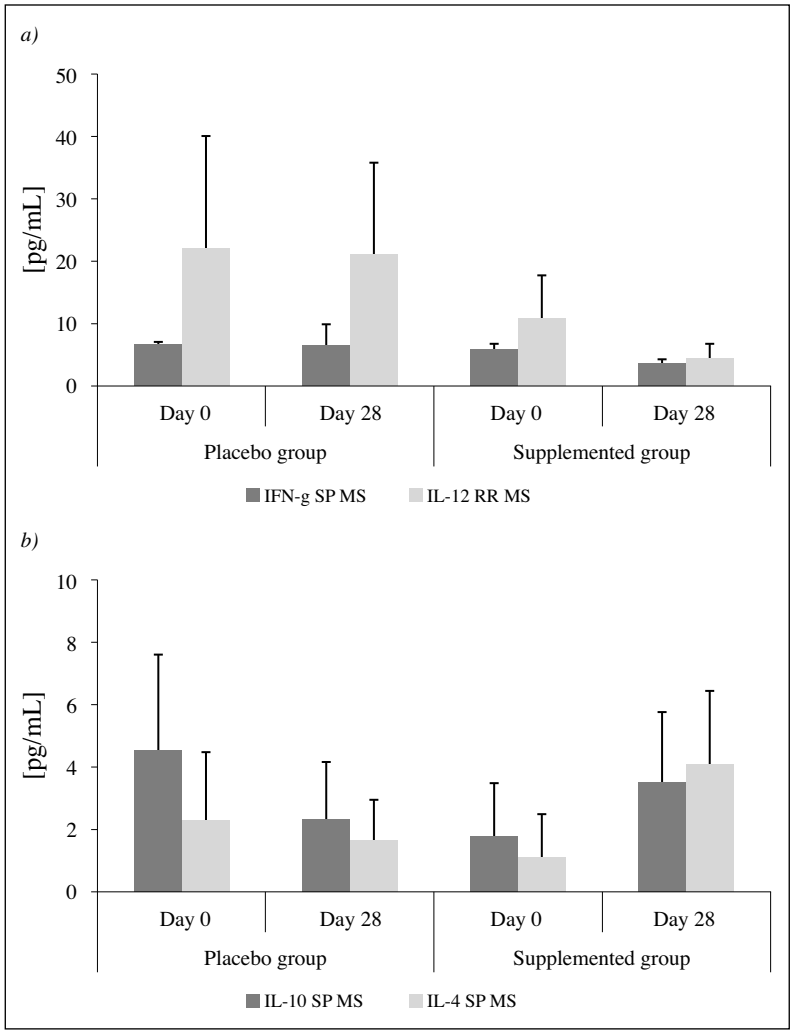

Fig. 1.—Representation of mean scores (SD) for: a) Pro-inflammatory cytokines IFN-g and Il-12 for MS and RR MS types respectively; and b) Anti-inflammatory cytokines IL-10 and IL-4 for SP MS type.
**Statistical significance ($p < 0.05$) according to the Student and/or Anova tests after intervention.*

comparing serum levels of CRP, oxidation products and inflammation biomarkers between healthy controls and MS patients[3,15]. The response to lemon verbena intake varied depending of the MS group. The effect of verbascoside supplementation on SP MS patients caused a significant decrease of CRP concentration, with a difference value of 7.1 mg/L ($p<0.003$) from baseline conditions. The results were in agreement with our previous work[16] thus confirming the impact of lemon verbena extracts containing verbascoside as anti-inflammatory supplement in diet interventions. No relation between lemon verbena administration and CRP serum levels was observed in RR and PP MS forms. The disparity in results may respond to the heterogeneity of inflammation activity inherent to each MS stage. Immunopathogenesis in MS involves higher CRP serum levels in patients with early age onsets (more associated with RR forms) compared to late age onsets more frequent in secondary progressive forms[17].

Therefore, the influence of verbascoside supplementation on the inflammatory response may be useful to control late exacerbations along disease progression[14].

Pro-inflammatory cytokines

Cytokines are well documented mediators of the autoimmune response in MS. The activation of CD4+ and CD8+ T helper (Th) cells primarily produce pro-inflammatory cytokines IL-2, TNF-α and IFN-γ (Th1) and anti-inflammatory cytokines IL-4, IL-10 (Th2) and TGF-β (Th3) whereas IL-12 and IL-23 are mainly expressed by monocytes and macrophages respectively. The binding of cytokines to their specific receptors triggers the phosphorylation of tyrosine residues and activates the immunological response[10].

The contribution of IFN-γ to the inflammation and the demyelinating processes involves the activation of mononuclear cells, the induction of the histocompatibility complex (MCH I and MCH II) and the differentiation and apoptosis of T cells[10]. The expression of IFN-γ increases with disease exacerbations and diminishes during remissions[18,19]. In our study, IFN-γ levels decreased after lemon verbena administration in all MS types, although no statistical significance was obtained for PP patients. Our findings are consistent

with previous published works that suggest the correlation between IFN-γ secreted levels and the MS clinical type. In particular, the IFN-γ response mediated by the T cell receptor seems to be increased in RR and SP MS patients[20]. Therefore, the supplementation of dietary supplements with anti-inflammatory properties such as lemon verbena extracts may affect the regulation of the signaling pathway by interfering with the IFN-γ role in MS pathogenesis.

The effect of lemon verbena supplementation on IL-12 serum levels also varied depending on the MS group. The intake of the supplement diminished significantly IL-12 concentrations in patients with RR MS while no effect was observed for the participants from the progressive groups. IL-12 functioning is associated with the differentiation of Th 1 cells[10,21] and its expression seems to be up-regulated in MS patients[22]. Particularly, IL-12 p40 levels were elevated in active MS lesions of patients with RR and SP presentations[23]. In addition, higher IL-12 (p70) serum levels were found in patients with acute MS forms[24]. The IL-12 (p70)/IL-23 quotient is considered as a potential biomarker to differentiate between SP and RR MS stages[25,26]. The correlation between IL-12 increased levels and the development of active MS lesions may explain the susceptibility of RR supplemented patients to the administration of anti-inflammatory compounds.

TNF-α is considered one of the most contributory factors to the immune mediated response in MS involving demyelination and oligodendrocyte damage[11,27,28]. TNF-α function includes the activation of cytokines, chemokines, mononuclear and adhesion cells. Similarly to other cytokines, TNF-α is up-regulated in MS patients and follows a positive correlation with the disease course[29]. Our results show higher baseline serum levels in all MS clinical subtypes while a similar decrease was found in progressive MS patients after lemon verbena supplementation in both placebo and intervention groups. These findings are consistent with several studies that reported elevated TNF-α levels during disease activity[30] and increased TNF receptors concentrations in patients with chronic progressive MS[31]. Consequently, the intake of the plant derived compound seems to cause no effect on TNF-α levels.

IL-6 and IL-23 response against lemon verbena supplementation was comparable to TNF-α results. IL-6 and IL-23 serum levels were increased for all MS groups at baseline and no statistically significant results were obtained regardless of dietary supplementation. The function of both cytokines is linked to the differentiation of TH 17 cells although IL-6 is also connected with the differentiation of B cells. IL-23 serum concentrations seem to be increased in RR patients[32,33], thus corresponding with our results before supplementation. Moreover, the expression of IL-6 cells is found to be inactive in demyelinating lesions with no association with disease activity or progression[34]. Accordingly no relation was found between anti-inflammatory supplementation and IL-6 or IL-23 cytokine decrease in all MS clinical subtypes.

Anti-inflammatory cytokines

IL-10 and IL-4 are down-regulatory cytokines produced by Th2 cells that inhibit the production or other cytokines. IL-4 expression is elevated in MS lesions during the relapse[35] while IL-10 levels are decreased before exacerbation in RR MS patients[36].

High serum levels were obtained at baseline for both cytokines in all MS groups with regard to normal ranges. For primary progressive presentations IL-4 and IL-10 concentrations increased for the intervention and the placebo group. Similarly, no significant differences were found in RR MS patients for both cytokines. In SP participants, IL-4 and IL-10 serum levels increased significantly ($p<0.05$) after supplementation. A comparable response measured as the increase of IL-4 expression has been reported for SP MS patients treated with anti-inflammatory drugs[37]. In contrast, decreased IL-10 levels have been associated with disease relapses in SP MS compared with RR patients. The elevation of IL-10 levels before the onset of exacerbations in RR MS may explain the variation of response observed only in the secondary progressive form.

The anti-inflammatory effect of TGF-β relies on the inactivation of macrophages and pro-inflammatory cytokines. Although TGF-β levels have been found to be increased after IFN-γ treatment, the supplementation of lemon verbena did not affect the concentration in serum since TGF-β decrease similarly in all groups.

The disparity of results reflects the limitation of nutritional interventions to study the impact of anti-inflammatory supplements on biochemical markers. To enhance the statistical significance of results future work should deal with the increase of the sample size and the development of feasible markers to monitor the response to inflammation. Nevertheless, the collected data comprises a relevant trend to approach the investigation of dietary supplementation on the cytokine profile in MS.

Conclusion

We present a placebo controlled study to assess the effectiveness of lemon verbena supplementation on inflammatory markers of multiple sclerosis patients. Our study tries to contribute to the low number of clinical trials regarding the effect of plant-derived compounds on inflammatory diseases. Results demonstrate that supplementation with lemon verbena extracts may affect the cytokine profile depending on the clinical subtype. Further investigation is needed to determine the specific role of pro-inflammatory and anti-inflammatory cytokines as mediators of the inflammatory

response on nutritional interventions. The increase of comprehensive studies will help to verify the efficacy of dietary supplements as complementary therapies in multiple sclerosis.

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