ERYTHROCYTE MEMBRANE FATTY ACIDS IN MULTIPLE SCLEROSIS PATIENTS AND HOT-NATURE DIETARY INTERVENTION WITH CO-SUPPLEMENTED HEMP-SEED AND EVENING-PRIMROSE OILS

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Abstract

The risk of developing multiple sclerosis (MS) is associated with increased dietary intake of saturated fatty acids. For many years it has been suspected that this disease might be associated with an imbalance between unsaturated and saturated fatty acids. We determined erythrocyte membrane fatty acids levels in Hot nature dietary intervention with co-supplemented hemp seed and evening primrose oils in multiple sclerosis patients. To determine the erythrocyte membrane fatty acids levels and correlate it with expanded disability status scale (EDSS) at baseline after 6 months intervention in MS patients by gas chromatography, in this double blind, randomized trial, 100 RRMS patients with EDSS<6 were allocated into three groups: "*Group A*" that received co-supplemented *hemp seed* and *evening primrose* oils with advised Hot nature diet. "*Group B*" received olive oil and "*Group C*" received the co-supplemented oils. The results showed that the mean follow-up was 180±2.9SD days (N=65, 23 *M* and 42 *F* aged 34.25±8.07 years with disease duration of 6.80±4.33 years). There was no significant difference in the study parameters at baseline. After 6 months, EDSS, Immunological parameters and the erythrocyte cell membrane with regard to specific fatty acids showed improvement in the group A and C, whereas there was worsening condition for the group B after the intervention. We concluded that Hot-nature dietary intervention with co-supplemented hemp seed and evening primrose oils caused an increase PUFAs in MS patients and improvement in the erythrocyte membrane fatty acids composition. This could be an indication of restored plasma stores, and a reflection of disease severity reduction.

Keywords: *Oenothera biennis L*, *Cannabis sativa L*, Polyunsaturated fatty acid, expanded disability status scale (EDSS), Cell membrane fluidity (CMF)

List of Abbreviation: AA, Arachidonic acid; ALA, Alpha-linolenic acid; CMF.Cell membrane fluidity;CNS, Central nervous system; D6D(FADS2),Delta -6-desaturase; DGLA, Dihomo-gamma-linolenic Acid; DHA, Docosahexanoic acid (key omega-3); EDSS, Expanded Disability Status Scale; EFAs, Essential fatty acids; EPA, Eicosapentaenoic acid; EP, Evening primrose; EPO, Evening primrose oil; FAs, Fatty acids; FAME, Fatty acid methyl esters; FDA, Food and drug administration; FR, Food Records; GC, Gas chromatography; GLA, Gamma Linolenic acid; HS, Hemp Seed ; HSO, Hemp Seed oil ; FN, Interferon (B1b-B1a-B) ; IFN-γ, Interferon- γ ; IL, Interleukin-4 ; LA, Linoleic acid (omega-6 family) ; LC-PUFA, Long chain- polyunsaturated fatty acid; MS, Multiple sclerosis; MUFA, Monounsaturated fatty acids; nqFFQ, non-quantitative Food Frequency Questionnaires; NSRC, Neurosciences Research Center; ω3-PUFAs, omega3-polyunsaturated fatty acids; PGE, Prostaglandin (E1, E2, E3); PUFA, Polyunsaturated fatty acid; RBCs; Red Blood Cells; RRMS, Relapsing Remitting Multiple sclerosis; SFAs, Saturated fatty acids; SDA (STA), Stearidonic acid; Th, T helper (1-2); TIM,Traditional Iranian Medicine; USFA,Unsaturated fatty acid; W/C, Warmth/Coldness

Introduction

Multiple sclerosis (MS) is an inflammatory disease of the central nervous system (CNS) of an unknown origin. Since the middle of the previous century, many investigations have tried to link MS incidence with dietary habits [Schwarz and Leweling, 2005]. The theory of Hot and Cold natures in people and foods finds its origin in ancient Greece, by Hippocrates (Greek physician, 460–375 BC) and Galen (199–129 BC) [Chiappelli et al., 2005; Ody, 1993; Ott, 1997]. Shahabi et al. (2008) showed the people of a Hot nature had more deviation of the immune system toward T-helper (Th2) responses than the subjects of a Cold nature, and in concordance with Traditional Iranian Medicine (TIM) practitioners' view that MS which is a Th1-mediated autoimmune disease occurs in persons of a Cold nature. Thus, the consumption of Hot Nature diet by persons suffering from an autoimmune disease with a deviation toward Th1 immune responses (such as MS) might be useful, as Shahabi's results show that this diet may shift the immune system towards Th2 responses. As there is evidence that omega-3-polyunsaturated fatty acids (ω3-PUFAs) can suppress IFN-gamma production in MS patients (Gallai et al., 1995), and hemp seed oil (HSO) contains these substances as well as evening primrose oil (EPO), the combination of these oils as a dietary supplement has a potentia to reduce pro-inflammatory cytokines and targets this key mechanism of disease and works like approved treatments (Yong et al., 1998; Rieks et al., 2003).

This would mean less deviation toward Th1 immune responses and may lead to a reduction in lipids peroxidation in the membranes. An ideal balance of omega6/omega3 (ω6/ ω3) should be 2.3:1. This ratio needs to be reached because these two groups of essential fatty acids (EFAs) perform distinct and complementary functions [Roncone et al., 2010]. Evidences showed that an increase in saturated fatty acids (SFAs) is associated with an increased risk of developing MS and increased EDSS, while increased PUFAs is thought to improve disease outcome [Van meeteren et al., 1981]. Diets deficient in EFAs to be associated with the cell membrane fluidity (CMF) influenced diseases; EFAs deficiency has been associated with MS [Rivers & Frankel, 1981]. CMF is determined by the constituents of the cell membrane. For example, an increase in membrane unsaturated fatty acids (USFAs) content or a decrease in membrane cholesterol will increase CMF [Rotten et al, 1973]. The maintenance of adequate levels of CMF is necessary for optimal cellular function [Scott, 1982], and the EFA is important in the active phase of the myelin synthesis [Auested, 2000; Salvati et al., 2000]. Thus, a shortage of dietary PUFAs may be a risk factor in MS. Indeed, later studies found decreased levels of both ω3 and ω6 PUFAs in red blood cells, plasma, and adipose tissue of patients diagnosed with MS [Holman et al., 1989]. We therefore designed a study to investigate the effects of a 9:1 combination HSO with EPO as a supplement to a Hot Nature diet in

comparison to the 9:1 combination of HSO with EPO without a special diet and olive oil in the third group. It might have an effect on erythrocyte membrane USFAs content

The ω6/ ω3 ratio in HSO is normally between 2:1 and 3:1, which is considered to be optimal for human health [Simopoulos et al., 2000]. HSO contains kinds of antioxidants that not only exhibit potent antioxidative properties for scavenging free radicals, but may also act on specific signaling pathways for regulating inflammatory responses [Matthaus and Brühl, 2008; Oomah et al., 2002; Hendriks et al.,1978; Nissen et al., 2009]. EPO is being used in increasing amounts in nutritional and pharmaceutical preparations, and may alleviate various chronic disease states [Horrobin, 1992; Huaug & Mills, 1995; Fan, 1998]. Whereas measurements of serum fatty acids (FAs) are heavily influenced by day to day changes in FAs ingestion, erythrocyte membrane FAs composition is a measure of the long term dietary FAs intake over the past several months**.**

This study is designed to assess the effects of the co-supplemented oils intervention with Hot nature dietary on erythrocyte membranes fatty acids composition of MS patients with clinical subtypes of RRMS.
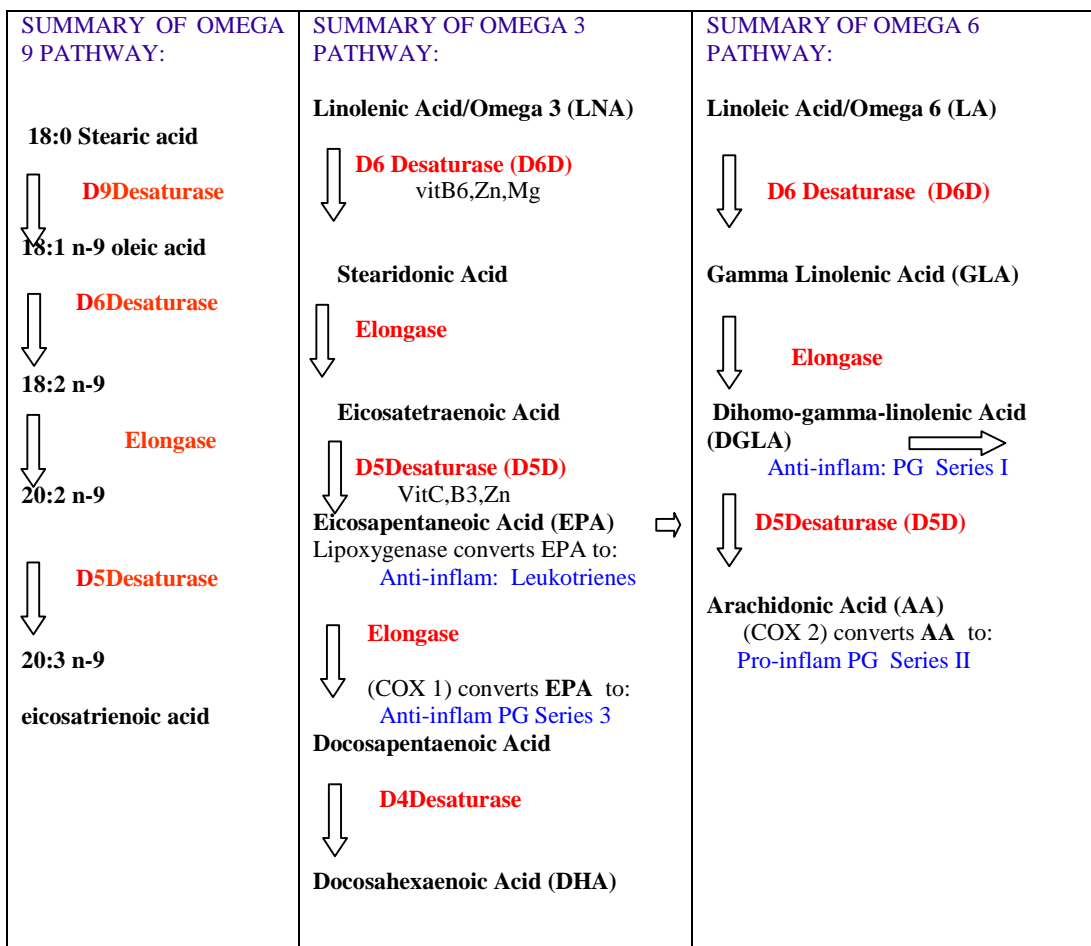

Material and Methods

This double-blind, randomized clinical trial was carried out on 100 RRMS patients who were allocated into three groups. The study was approved by the Neurosciences Research Center (NSRC) and local ethics committee of Tabriz University of Medical Sciences. MS patients were recruited through the MS Society. Patients with a definite diagnosis of MS using the Kurtzke EDSS <6 criteria [Kurtzke, 1983] with type of RRMS, ages 14-55 years, were enrolled. Patients with secondary or primary progressive MS, pregnancy, corticosteroid treatment, and patients who suffered concomitantly from another chronic disease such as rheumatic diseases, serious heart diseases, malignant tumors, and other neurological and inflammatory illnesses were excluded. Patients were allowed to continue their routine medications [only Interferon: Avonex one time/week] (Table 1). A written informed consent was completed prior to the study for all patients. The patients completed a 3-day food record in the first and the last week, a non-quantitative Food Frequency Questionnaire (nqFFQ) to assess food and drinks consumed and dietary habits. They were asked to maintain their usual level of physical activity and not to consume any supplements during the study. We must notice that the co-supplemented oils (combination of *hemp seed oil* and *evening primrose oil* with 9/1 ratio*)* are foodstuffs and without side effects. To reduce or eliminate a number of factors possibly affecting the measured results, random block design was used by a satiation who was not involved directly in the trial. Then, the patients were randomly assigned into three groups to receive one of the three dietary interventions:

"*Group A*": those who received the co-supplemented oils, 18-21g/day (6-7g, three times daily) with advised *Hot nature diet*,
"*Group B*": those who received olive oil 18-21g/day (6-7g, three times daily),
"*Group C*": those who received the co-supplemented oils, 18-21g/day (6-7g, three times daily) for 6 months.

To achieve this objective, group A was asked to consume "*Hot nature diet*" with a wide choice of food and drink items permitted during each dietary period and delivered at home for 6 months [**APPENDIX A**].

Appendix A**:** The Polyunsaturated Fatty Acids biosynthetic pathway

SUMMARY OF OMEGA 9 PATHWAY:	SUMMARY OF OMEGA 3 PATHWAY:	SUMMARY OF OMEGA 6 PATHWAY:
18:0 Stearic acid	**Linolenic Acid/Omega 3 (LNA)**	**Linoleic Acid/Omega 6 (LA)**
⇩ **D9Desaturase**	⇩ **D6 Desaturase (D6D)** vitB6,Zn,Mg	⇩ **D6 Desaturase (D6D)**
18:1 n-9 oleic acid	**Stearidonic Acid**	**Gamma Linolenic Acid (GLA)**
⇩ **D6Desaturase**	⇩ **Elongase**	⇩ **Elongase**
18:2 n-9	**Eicosatetraenoic Acid**	**Dihomo-gamma-linolenic Acid (DGLA)** ⇨ Anti-inflam: PG Series I
⇩ **Elongase**	⇩ **D5Desaturase (D5D)** VitC,B3,Zn	⇩ **D5Desaturase (D5D)**
20:2 n-9	**Eicosapentaneoic Acid (EPA)** ⇨ Lipoxygenase converts EPA to: Anti-inflam: Leukotrienes	**Arachidonic Acid (AA)** (COX 2) converts **AA** to: Pro-inflam PG Series II
⇩ **D5Desaturase**	⇩ **Elongase** (COX 1) converts **EPA** to: Anti-inflam PG Series 3	
20:3 n-9	**Docosapentaenoic Acid**	
eicosatrienoic acid	⇩ **D4Desaturase**	
	Docosahexaenoic Acid (DHA)	

[**APPENDIX A**] **Permissible foods** :Hot nature diet for Group A
the low rate of catena & rice - **Cereals and grains:** wheat bread –beans-peas-cotyledon –soya products- wheat germ in soup-macaroni with Soya or mutton-dry wheat germ-macaroni-variety of wheat bread without adding- potatoes puree with milk -forage of meat or HALIM-the low rate of rice- wheat germ porridge
Meat and eggs :goose - turkey- mutton-veal-quail -domestic poultry and rooster-shrimp-liver-heart-tongue-brain-variety of south and north fishes(salt water fishes)-caviar-white of the egg
concentrate yogurt–shallot mixed **Dairy products :**fresh milk honey -cream honey –kinds of cheese with walnut or dates-concentrate yogurt- Kefir yoghurt
Fats :olive oil-grape seed oil-sesame oil
Sweets :grape juice-brown sugar-sugar candy with rose water-honey-sesame pudding, SAMANO(it is a kind dish with juice of germinating wheat or malt mixed with flour)-rose jam- orange flower jam-walnut jam
Vegetables : kinds of cabbage-shallot-celery-tarragon-mint-cress- radish- garlic-onion-carrot-kinds of pepper- sweet basil - corrugated variety of cucumber -clover-fenugreek-coriander-savory -grape leaves-turnip-dill-mushroom-pumpkin-spring onion-pennyroyal-parsley-beetroot-eggplant- tomato(low)
Fruits :cantaloupe-olive-black olive-grape-fig-sweet pomegranate-cherry-dates-coconut-banana-mango-pine apple-quince-grapes- apple-berry- -pear-melon-sweet citreous
Spice :turmeric-mustard-cinnamon-caraway-ginger-saffron-green cardamom-pepper-vanilla-cocoa powder- nigella seeds-tomato paste (low)-pomegranate paste (low) -lemon juice (low)
Nuts: various nuts without additives –pea nut-Indian almond – walnut- sweet almond-pistachio-hazel nut- Soya nut without additives - sun dried grapes- sun flower seeds-sun dried apricot -sesame-melon seed-linseed -pumpkin seed-water melon seed
Drinks :tea-green tea- the tail of some vegetable- cool drinking with sweet basil -orange flower –mint- fennel- sweet basil-bee balm and borage water alfalfa
Different kind of foods prepared by traditional methods of the above materials

Groups "B and C" were asked to consume their usual diet during the intervention. *"Hot nature diet"* includes foods with Hot nature, low intake of cholesterol, hydrogenated or trans fatty acids and saturated fats (fried foods), the consumption of olive or grape seed oils as main oils in daily diet, eating plenty of fresh fruits and vegetables with Hot nature, nuts and seeds without additives, fish and seafood, unrefined carbohydrates, drinking plenty of water (avoiding too much drink containing artificial additives, sweeteners or other stimulants), cutting down sugar and refined starch (i.e. non-whole meal bread, cakes, pastries, biscuits, sweets and soft drinks), consumption of dairy products with honey or date and removing foods with *Cold nature* [**APPENDIX B**],

[**APPENDIX B**] **Impermissible foods** :Clod nature diet for Group A
Cereals and grains: Rice-lentils-vetch-potato-starch-barley bread-corn-bean broad
Meats : Beef-machine chicken- fishes live in river- egg yolk- SIRABI(sheep's leg & intestine) -chicken liver – canned fishes -processed meat-sausage type-hamburger
Dairy products : Dairy without walnut or dates- sour Dughe (sour yogurt diluted with water) - milk powder-whey-different kind of ice cream
Fats :solid suet- natural butter and liquid vegetable fats-fats link to meat and poultry-palm oil
Sweets : Zoolbia Confectionary type Bamie-junk foods including types of toffee, candy ,chocolate ,chips ,snack
Vegetables : Rhubarb-lettuce-cucumber- spinach-green beans-green peas- green bean broad-okra-beetroot leaves
Fruits : Peach-strawberry- nectarines- meddler-water melon-kiwi-greengage-sour pomegranate-blue berry-sour citrus-sour cherry-rhubarb-sour fruits-plum
Spice : unripe grapes-unripe grapes juice-different sauce-tamarind- salty foods- Sun dried fruit (LAVASHAK) -sumac-sorrel
Nuts: dried with sulfur-sulfur raisin-salty and spicy nuts
Drinks : Nonalcoholic Beer -soft drink and alcoholic- sour Dughe
Fried foods- canned and semi canned foods-different kind of sandwich and pizza and other fast foods-fermentation foods

avoidance of fast foods, alcohol and smoking. To prevent bias at several stages of the trial, the patients, the investigators and assessors particularly neurologist who assess EDSS score were unaware and blinded of the assigned intervention. The patients were contacted monthly by telephone to assess compliance. After baseline assessments, 100 patients were randomized to three groups according to following diagram (Figure. 1).

All measures were repeated similarly with same approach and assessors at the end of intervention period. Researcher, patients and those involved in the data collection and assessment (neurologists and nutritionists) as well as data analysis were blind regarding the type of interventions.

Measurement of the disability status of the patients

Change in EDSS was used as secondary outcome measures. The functional disability status (disease severity) of each patient was measured by a trained clinician using the Kurtzke EDSS [Kurtzke, 1983]. Scales for the total EDSS are from 0 to 10, in which the 0 score indicates no disability at all and 10 indicates death due to MS.

Blood sample processing and analysis

Venous blood samples (10 ml) were collected from the patients before and 6 months after treatment. Red blood cells (RBCs) were washed in a 0.85% saline solution and immediately transferred to small glass vials, layered with nitrogen, and stored up to one year at -80°C. Total lipids

were extracted from RBCs with chloroform/ methanol (1:2v/v), then fatty acids were separated from their alcohols and etherified by methanolsis to form fatty acid methyl esters (FAME). FAME was injected in gas chromatography and analysis fatty acids composition [Van Jaarsveld et al., 2000; Folch et al., 1957]. GC is the standard measurement technique for the assessment of fatty acids in biological tissues. Red blood cells FAs were quantified in μg FA/ml packed RBC. The cytokine assay for IL-4 and IFN-γ was performed using the enzyme-linked immunosorbent assay (ELISA) with techniques commercially available kits (U-CyTech. Netherlands). The absorbance of each well was read at 450 nm.

Statistical analysis

The statistical analysis was performed using SPSS software (ver 14.0; SPSS Inc, Chicago, IL). Data were expressed as mean ± standard deviation (SD). Pre- and post within each intervention comparison in continuous variable were done using paired t-test. Statistical significance was defined as $p < 0.05$.
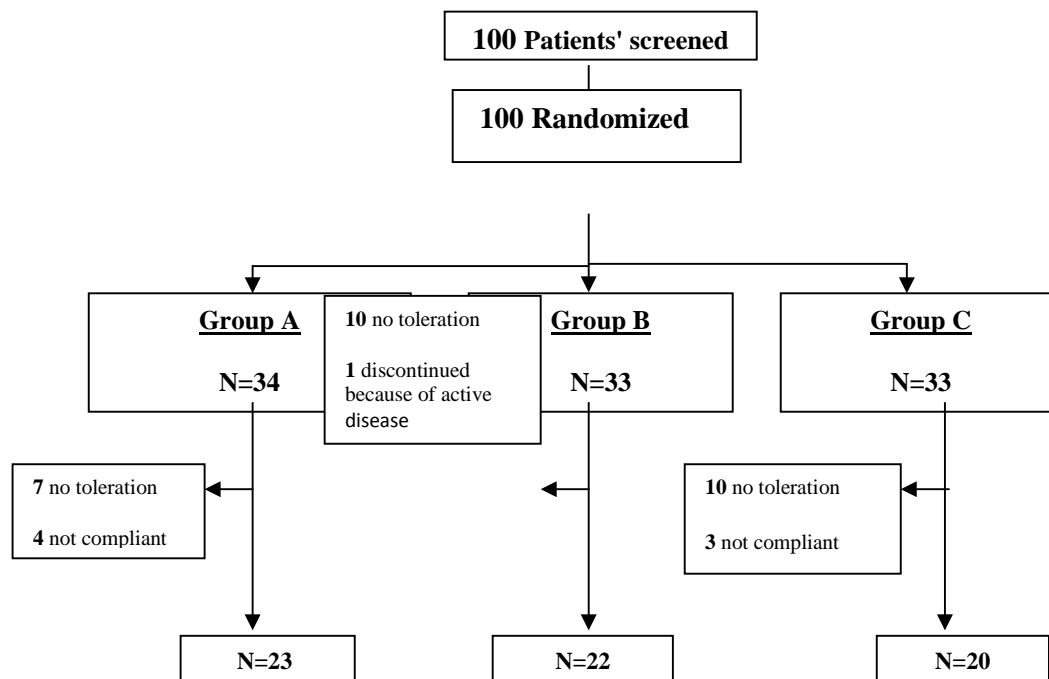
Figure 1: Flowchart of the study; 100 patients were randomized to three groups; group A: Co-supplemented hemp seed and evening primrose oils and advised Hot nature diet; group B: Olive oil; group C: Co-supplemented hemp seed and evening primrose oils.

Results
Characteristic and demographic results in RRMS patients

One hundred (34 M and 66 F) patients were enrolled in this study. Figure. 1 summarizes the patient attrition patterns in the study. The dropout rate was 35 from 100 patients (11 in "group A", 11 in "group B", and 13 in "group C"). This study was performed between October 2010 and October 2011. The patients' characteristics and demographics are shown in (Table 1). The sample consisted of 23 males and 42 females with a mean age of 34.25±8.07 years and mean disease duration of 6.80±4.33 years. There was no significant difference in the mean gender, disease duration, interferon intake, age, and average age at onset between the treatment groups.

Tables of clinical and biochemical results in RRMS patients

Table 1: Clinical and demographic characteristics of the study patients n=65 (23 men, 42 women).

Variable	Group A (N=23)	Group B (N=22)	Group C (N=20)
	Mean ± SD	Mean ± SD	Mean ± SD
Age(years)*	34.2±7.5	35.9±7.8	33.7±7.8
Average age at onset (years)*	25.0±7.5	30.3±8.1	27.6±6.4
Disease duration (years)*	6.26±3.9	7.55±5.08	6.60±4.0
	N (%)	N (%)	N (%)
Interferon intake	22(95.7)	22(100)	19(95)
Gender (M/F)	7/16	11/11	5/15

Group A: Co-supplemented hemp seed and evening primrose oils and advising Hot nature diet
Group B: Olive oil
Group C: Co-supplemented hemp seed and evening primrose oils.

Result of Table 2 indicates the effects of the interventions on IL-4, IFN-*y* and over the study in three groups. A trend in the decrease of mean pro-inflammatory cytokine IFN-γ was observed in group A, while anti-inflammatory cytokine IL-4 concentration increased significantly after 6 months in groups A and C which indicates decrease of inflammation in groups A and C. IFN-γ in group B increased significantly and in turn resulted in significant changes in their concentration among the groups. The clinical results of the trial are summarized in Table 2. There were significantly better changes in EDSS in groups "A and C" at the end of the intervention, while olive oil consumption resulted in a significant increase in EDSS.

Table 2: Effect of intervention on mean (±SD) immunological factors: Interleukin-4 (IL-4), Interferon- *y* (IFN- *y*) and Expanded Disability Status Scale (EDSS) in trial groups of RRMS patients; comparison to baseline

Trial Groups	Group A (N=23)			Group B (N=22)			Group C (N=20)		
Variables	**Baseline**	**6 months**	**P**	**Baseline**	**6 months**	**P**	**Baseline**	**6 months**	**P***
IL-4	.58±.50	.69±.69	**.027**	.50±.50	.41±.14	.310	.81±.87	.95±.91	**.046**
IFN-γ	.26±.04	.24±.04	**.001**	.22±.06	.24±.06	**.005**	.35±.23	.31±.14	.079
EDSS	2.76±1.39	1.7±1.77	**0.001**	3.45±1.41	3.86±1.41	**.005**	3.25±1.94	2.95±1.83	**0.002**

*P for paired-t test

The absorbance of cytokines levels was read at 450 nm.
Group A: Co-supplemented hemp seed and evening primrose oils and advising Hot nature diet
Group B: Olive oil
Group C: Co-supplemented hemp seed and evening primrose oils.

Table 3 indicates significant differences in relative SFA, MUFA, PUFA, between cases groups' patients. In group A, the MS patients had significantly higher overall levels of PUFAs, eicosapentaenoic acid (EPA) and arachidonic acid (AA), and also, lower myristic acid and omega-9 fatty acids. In group C, the MS patients had significantly higher levels of PUFAs and palmitoleic acid, in addition, lower α-linolenic acid (ALA) and MUFAs. Whereas, the patients in group B had significantly lower overall levels of eicosapentaenoic acid (EPA), ALA and MUFAs. The overall level of SFAs was also significantly higher for patients in group B. The other differences were not significant, but was the overall difference in PUFAs after adjusting for multiple comparisons. With regard to specific FAs, in group A, the patients had significantly better values in comparison with groups "B and C". There were no serious adverse effects in any of the 65 MS patients.

Table 3: Effect of intervention on mean (±SD) red blood cell membranes fatty acids composition in trial groups of RRMS patients comparison to baseline

Variables	Group A (N=23)			Group B (N=22)			Group C(N=20)		
	Baseline	**6 months**	**P**	**Baseline**	**6 months**	**P**	**Baseline**	**6 months**	**P**
14C:0	1.71±.69	1.29±.61	**.050**	1.44±.70	1.45±.77	.982	1.17±.77	1.03±.42	.630
16C:0	34.77±3.84	33.39±6.15	.468	33.82±3.95	36.49±2.98	**.050**	38.32±3.42	36.42±3.09	.083
16C:1n7	.90±.41	.79±.61	.604	.97±.57	.97±.57	.997	.95±.81	1.22±1.02	**.050**
18C:0	15.26±1.56	15.93±1.81	.138	14.82±1.90	14.97±1.59	.544	15.36±1.80	15.96±1.29	.080
18C:1n9 Trans	.23±.17	.26±.19	.675	.31±.32	.19±.10	.212	.17±.09	.24±.16	.143
18C:1n9	11.28±1.76	9.27±2.13	**.000**	11.49±2.28	8.76±.93	**.001**	10.23±1.43	8.55±.76	.001
18C:2n	21.70±2.53	23.05±2.88	**.040**	21.66±3.88	22.22±3.23	.473	19.05±2.37	20.61±1.61	.058
20C:0	1.00±.57	.89±.48	.236	1.05±.66	.89±.48	.078	.57±.32	.65±.29	.129
18C:3n	.16±.13	.17±.21	.792	.19±.07	.09±.05	**.000**	.15±.10	.10±.04	**.032**
20C:4n6	10.58±2.80	12.63±2.09	**.023**	11.83±2.26	11.68±1.99	.806	11.64±2.41	12.80±2.10	.152
EPA	.88±.41	.99±.37	**.046**	.82±.45	.62±.36	**.015**	.76±.24	.68±.21	.152
DHA	1.59±.56	1.53±.71	.515	1.60±.44	1.68±.27	.444	1.67±.52	1.73±.35	.514
SFA	52.73±3.81	51.50±5.11	.434	51.13±3.97	53.79±2.92	**.050**	55.43±3.35	54.06±2.75	.166
MUFA	12.40±1.78	10.33±2.28	**.000**	12.77±2.26	9.92±1.15	**.000**	11.35±1.61	10.02±1.44	**.002**
PUFA	34.87±4.01	38.17±3.26	**.013**	36.10±4.31	36.30±3.78	.877	33.22±3.71	35.92±2.38	**.028**

- **P for paired-t test**
- **RBC FAs quantified in µg FA/ml packed RBC**
- **Names of fatty acids examined in this study :**

14C:0;Myristic acid ;16C:0;Palmitic acid ; 16C:1;Palmitoleic acid ; 18C:0;Stearic acid ; 18C:1trans:Trans-Oleic acid ; 18C:1;Oleic acid 18C:2;Linoleic acid ; 20C:0;Arachidic acid ; 18C:3; (α-Linolenic acid, Stearidonic acid, Gamma Linolenic Acid); 20C:4;Arachidonic acid 20C:5n3; EPA:Eicosapentaenoic acid; 22C:6n3 ;DHE:Docosahexaenoic acid; SFA:Saturated fatty acids; MUFA:Monounsaturated fatty acids; PUFA:Poly unsaturated fatty acids; Group A: Co-supplemented hemp seed and evening primrose oils and advising Hot nature diet; Group B: Olive oil; Group C: Co-supplemented hemp seed and evening primrose oils.

Discussion

Traditional Iranian Medicine (TIM) roots go back over 2000 years [Naseri, 2004; Naseri and Ardakani, 2004]. Cold and Hot natures in people and foods have been believed to exist in TIM and also in many other traditional medical theories. The most important piece of work, carried out in the East, was the book *Canon of Medicine* (*Qanun dar Tib*), written by Avicenna [Avicenna, 2004; Ody P, 1993; Ott J, 1997]**.** Immune responses are divided into two groups: T-helper (Th1) and (Th2). Cold and Hot natures theory says that a person with a very Hot nature has susceptibilities to allergic reactions and immune responses toward Th2-like responses (ex; interleukin IL- 4) and a person with a very Cold nature (autoimmune disease such as MS) has immune responses toward Th1-like responses (ex; interferon IFN-γ) [Shahabi et al, 2008]. Cold and Hot natures theory may confirm the importance of host and food factors in determining the type of response to stresses. It means that the nature of a people and their foods can also be important in connection with disease. The consequences of a person's exposure to acute or chronic stresses may be influenced by the nature of the person [Shahabi et al., 2008]. This concept can be one of the causes (in addition to others) of differing susceptibilities to specific diseases between different people [Kaplan et al., 2001]. Based on TIM practitioners' view, MS is a Th1-mediated autoimmune disease is more prevalent in Cold nature persons [Shahabi et al., 2008], so consumption of Hot nature foods may be useful because they can accelerate warmth of nature and deviation toward Th2 immune responses and may lead to a reduction in disease severity [Mirzaei, 2007].

In this study, immunological assay confirmed the results of clinical examinations, depending on Tables 2, which indicated that groups A and C and special group A had a higher rate of deviation of the immune system toward Th2 responses and were healthier in comparison with group B, while a hallmark in the pathogenesis of MS was a shift in the ratio of Th cells towards Th1 cells. It may explain why therapies that promote a Th1 to Th2 cytokine-shift are beneficial in MS patients [Sasa sega et al., 2004]. Epidemiological studies have demonstrated a relation between MS mortality and dietary fat [Esparza et al, 1995]. Lipids serve important functions as membrane phospholipids constituents [Huwiler and feilschifter, 2009]. Analysis of SFAs/USFAs ratios between normal subjects and MS patients have showed statistically significant increase in MS patients and an increase of SFAs may explain the well-known changes in MS patients [Shore and Alpers , 1963; Caspary et al.,1965; Wright et al., 1965]. In combination, changes in cytokine production may provide prolonged changes in inflammatory responses relative to the rapidly re-equilibrating levels of PUFA and their metabolites. Dietary PUFA affect inflammatory functions and anti-inflammatory cytokine production from mononuclear cells affect lipids peroxidation proceed. Several clinical observations, suggests that abnormalities of PUFAs synthesis may be involved in MS [Ghadirian et al., 1998].

In addition, if EFA deficiency occurs during the postnatal period, a major delay in the myelination process will occur, accompanied by impaired learning, motor, vision, and auditory abnormalities [Stockard et al, 2000]. If EFA are not available in this phase or are metabolically blocked, amyelination, dysmyelination, or demyelination may occur [Auested, 2000; Salvati et al., 2000], and the age of susceptibility decrease. In this way the correct diet should be kept from pregnancy and child development period. We supposed that the combination of *hemp seed oil (HSO)* and *evening primrose oil (EPO)* [as co-supplemented oils] intervention with advising Hot nature diet has effect increase in erythrocyte membrane USFAs content. The *hemp seed (Cannabis sativa L)* has been used as a food /medicine in China for at least 3000 years [De Padua et al, 1999]. HSO has over 80% in PUFAs, and ω6/ ω3 ratio in 2:1 and 3:1, which is considered to be optimal for human health [Simopoulos et al., 2000], and this reflects the ratio found in the traditional Japanese and Mediterranean diets [Callaway et al., 2004; Kriese et al., 2004]. By the presence of gamma linolenic acid (GLA), oil of *evening primrose* (*Oenothera biennis L*) is being used to increase amounts in nutritional and pharmaceutical preparations, and may alleviate various chronic disease states [Horrobin, 1992; Huang and Mills, 1995; Fan and Chapkin, 1998] and using of *EPO* which is a popular alternative treatment for MS in some countries. HSO has between 80 and 110 mg/100 g tocopherols (α-, β-, δ-, γ-tocopherol) with γ-tocopherol as the main tocopherol (85%) [Matthaus and Brühl, 2008; Oomah et al., 2002]. Since USFAs are highly susceptible to peroxidation, an increased intake of these agents without anti-oxidant protection might produce the undesirable effect of decreasing CMF through peroxidative cross linking reactions in the cell membrane [Slater, 1982]. The 'free radical' theory of aging [Barber and Bernheim, 1967] accords well with the fact that lipid peroxidation leads to decreases in CMF [Vladimirov et al., 1980]. HSO contains phytosterols, terpenes and kinds of tocopherol that not only exhibit potent antioxidative properties for scavenging free radicals, but may also act on specific signaling pathways for regulating inflammatory responses [Matthaus and Brühl, 2008; Oomah et al., 2002; Hendriks et al., 1978; Nissen et al., 2009]. CMF is a parameter crucial to the maintenance of cellular function. Alterations in CMF are seen in several disease processes and the normalization of CMF in these diseases may prove therapeutic. CMF appears to influence several cellular processes including the activity of membrane-associated enzymes [Dobretsov et al., 1977; Schroeder et al., 1976], the availability of membrane receptors [Knazek and Liu, 1979] and events occurring during the course of the cell cycle [Lai et al., 1980]. The activity of membrane-associated enzymes increases in more fluid membranes [Dobretsov et al., 1977]. Cell division is associated with changes in CMF, greatest fluidity occurring during mitosis [Lai et al, 1980]. Finally, the membrane fluidity index or CMF is a common denominator for the various effects of the various PUFAs and ω6/ω3 ratios [Yehuda, 2003; Yehuda et al., 2000]. The rate of myelin lipids turnover is age dependent, and with a very slow turnover rate during aging, the rate of repairing damaged sections of myelin is correspondingly slower [Ando et al., 2003]. Also, CMF may also be implicated in the changes associated with the aging process. According to TIM practitioners' view, Hot nature and CMF in children are in the highest level that both decrease with age. Cold nature overcomes with elevation of age. Age-associated lowering of Delta-6-desaturase (D6D or FADS2) activity will decrease PGE1 synthesis [Horrobin, 1981]. D6D is the rate-limiting step in the PUFAs biosynthetic pathways that are incorporated into cell membranes, thereby affecting permeability and functional properties of cells (Appendix C). GLA is produced in the body from desaturation of linoleic acid (LA) by the reaction catalyzed by enzyme D6D. The presence of both GLA and stearidonic acid (SDA) in HSO, typically at a favorable ω6/ ω3 ratio of 2:1 that from a nutritional point of view, up to 7% GLA and 2.5% SDA is very interesting and allows this enzymatic step with D6D to be efficiently bypassed [Okuyama et al., 1997]. GLA and SDA are products of this enzyme delivering to the patient organism by the co-supplemented oils in this trial. These mentioned basic components for cellular metabolic pathways could easily replace this intervention in patients' organism A and C groups. It was found that a 9:1 HSO with EPO combination with and without Hot Nature diet led to a significant reduction in the EDSS score. Also, GLA and SDA may help to displace AA from membrane phospholipids that compete with AA for cyclooxygenase and lipoxygenase pathways (the mechanism behind NSAIDs). Of importance is that AA is a precursor of pro-inflammatory and pro-aggregator Prostaglandin (PGE2), but EPA and dihomo-gamma-linolenic acid (DGLA) are precursors of anti-inflammatory PGE3 and PGE1 series, respectively [Mitchell, 1992], and can be

enhanced by GLA and SDA. Both are rapidly and readily incorporated into cell membrane phospholipids [Lassmann, 1999; Kornek and Lassmann 2003] (according to Appendix C). Increasing evidence indicates that immune responses during infancy and early childhood are dominated by Th2 cytokines, but the shifting toward Th2 pattern decreases with age [Adkins et al., 2001; Holt et al., 2000], and this is in agreement with TIM's belief that the nature is dominated by Warmth at birth but its Warmth is accompanied with a level of CMF decreases with age [Avicenna, 2004]. In this way, diet with Hot nature can accelerate warmth of nature and deviation toward Th2 anti-inflammatory responses, and the co-supplemented oils increased CMF with increased EFAs and USFAs in membrane. It is another proof for the importance of our claim for the importance of our dietary intervention that no MS disorder happen during primary part of life and subjects with MS develop this disease in the period of their life which is accompanied with their dramatic changes in their diet. In this study, we analyzed RBC total FAs in three groups of MS patients at the base line and 6 months after intervention. We showed that in the case of groups "A and C", patients increase in EFAs and PUFAs in RBC membranes; whereas, group B showed a decrease in PUFA and an increase in SFA levels (Tables 3). Increases of EFAs or PUFAs ratio as well as with decrease in EDSS were significantly better in groups "A and C" compared to group B, and felt physically healthier (Tables 2). In this way, the co-supplemented oils suggested that a healthful balance of ω6/ω3 (2:1) FAs leads to modulate overall membranes FAs composition and may help reduce the risk of MS. Alterations in the proportions of various FAs classes in our study were showed in group A, patients had significantly better values in comparison with groups "C and B". Furthermore, increases in SFAs and/or MUFAs have reported to replace plasma and/or RBC membrane PUFA deficiencies [Holman et al., 1989; Cherayil, 1984; Navarro and Segura 1989], and this fact is also present completely in this trial (Tables 3). These results are in agreement with the complications relating to Hot or Cold nature of food dominance, and they indicate that the intensity of Warmth/Coldness of nature foods has different effects on the erythrocyte membrane fatty acid composition of MS patients associated with inflammatory responses. It is highly possible that Hot nature diets accelerate anti-inflammatory responses in patients and prevent pro-inflammatory cytokine production (Table 2), and equally help in the maintenance of fatty acids membrane. As well, the co-supplemented oils increased EFAs and USFAs in membrane group A of MS patients and caused an optimal balance between SFAs and USFAs. This might hinder the disease from progressing as observed increase in PUFAs in this study could be a mechanism against development of MS.

Conclusion

In summary, our data demonstrated that the co-supplemented oils with Hot nature dietary intervention may decrease the risk of developing MS. This finding led to the hypothesis that this intervention may have a specific improvement effect on erythrocyte membrane fatty acids composition and likely on mitochondrial and myelin membranes.

Future directions

Based on TIM practitioners' view, we could examine CMF, delta-6-desaturase and inflammatory factors (Th2 /Th1 ratio) in all groups of MS patients and healthy adults, and assay these parameters before and after Hot nature dietary intervention with co-supplemented oils. It is likely that the changes in lipid biology identified in multiple sclerosis with this intervention may be relevant to other psychiatric conditions and more generally to other neurodegenerative disorders.

Limitation

Uncontrolled diet is the other important confounding factor.

Acknowledgements

Conflicts of interest

The authors declare that they have no competing interests.

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