Original article

The effects of n-3 fatty acids on inflammatory cytokines in osteoporotic spinal cord injured patients: A randomized clinical trial

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Background: Clinical studies have reported that osteoporosis after spinal cord injury (SCI) can be the inflammation-induced base condition and n-3 polyunsaturated fatty acids (PUFAs) suppress the production of pro-inflammatory cytokines. This study documents the effects of n-3 PUFAs on cytokines in a group of patients after chronic SCI. **Methods:** This double-blind, placebo- controlled trial was designed in 82 (69 males and 13 females) osteoporotic patients with SCI for 4 months. All participants received 1000 mg calcium and 400 IU vitamin D daily. The patients received two MorDHA capsules (435 g of DHA and 65 mg of EPA per day) or two placebo capsules (one with lunch, and the other with dinner) in the treatment and control groups, respectively. Serum interleukins and Dietary intakes were assessed in the beginning and end of the study. Mean difference for each group was compared by using Student's *t* test. **Results:** A total of 75 (13 females, 62 males) participants completed the study over 4 months. The supplemented and control groups did not show any difference in their baseline characteristics. There were significant difference neither between two groups at the end of the study nor in each group between beginning and end of the study. **Conclusins:** MorDHA supplementation for 4 months had no significant effect on inflammatory markers. Although mean difference in all pro-inflammatory cytokines were not significant in both treatment and control groups during the study ($P>0.05$), the decrease in treatment group was weakly higher that it may be important in point of clinical view.

Key words: Cytokines, inflammation, omega 3 fatty acids, osteoporosis, spinal cord injury

INTRODUCON

Osteoporosis is a common disease that can affect persons with spinal cord injury (SCI).[1-4] Several clinical studies have reported that osteoporosis after SCI can be the inflammation-induced base condition. Moreover, interleukins (IL-1, IL-6) and tumor necrosis factor (TNF-α) were also able to stimulate bone resorption, and prostaglandin E2 (PGE2) is an important factor in mediating the effects of these cytokine.[3,5-7]

PGE2 is one of the important mediators that can stimulate inflammation-induced bone resorption markers. Therefore, PGE2 could stimulate the release of these cytokines from various mesenchymal cell lines.[6]

Many studies show that n-3 polyunsaturated fatty acids (PUFA) enhance bone formation and suppress the production of pro-inflammatory cytokines like tumor necrosis factor (TNF-α) and interleukin (IL-1β and IL-6) in human and animal models.[5,8,9]

In a previous and unique interventional study,[10]Javierre *et al.* evaluated the possible effect of n-3 PUFA (1.5 g of DHA and 0.75 g of EPA) on plasma lipid profile in 19 adult males with SCI for 6 months. However, there were no observable differences in lipid profile during the study.

SCI per se is associated with a low-grade chronic inflammatory state.[2,11] All of human studies that evaluated the effect of n3 PUFA on inflammation markers have focused on different diseases, but to the best of our knowledge, there is no study about the effect on these nutrients in SCI patients. So, we designed this

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study to evaluate the possible effect of n-3PUFAs on pro-inflammatory cytokine bone change in chronic spinal cord injured patients.

METHODS

Study samples

This double-blind, placebo-controlled trial (138905102709N7: August 28, 2010) is designed in two groups in Brain and Spinal Injury Repair Research center (BASIR) from our previous cross-sectional studies.[12,13] We selected 75 (69 males and 13 females) persons who were willing to participate in the study. Patients who met the initial criteria during a phone screen entered the study. Individuals were included for study according to the following criteria: traumatic spinal cord injured patients, 18 years of age or older, 1-year post injury. Required data were collected in the BASIR Research Center at Tehran University of Medical Sciences (Tehran, Iran) from January 2011 to July 2011. Anthropometric measurements and baseline blood sampling of the participants were evaluated after giving the informed consent. The protocol was approved by the ethics committee at Tehran University of Medical Sciences (Approval number: 1421 at July 18, 2010).

Osteoporosis was diagnosed by evaluating BMD with dual energy X-ray absorptiometry at femur and lumbar vertebrae. BMD was measured by DXA using Lunar DPX-MD device (Lunar Corporation, Madison, Wisconsin, 53713, USA).[14,15] The calibration for instrument was done weekly by using appropriate phantoms. The precision error for bone mineral density measurements was 2 to 3 in the femoral and 1 to 1.5 in the lumbar regions. All scans were performed according to the manufacturer's guidelines. BMD was determined using DXA method. Osteoporosis was defined as T score ≤-2.5 in femoral neck region.[14] All study participants were medically stable upon inclusion into the study. The participant's demographic data including sex, age, possible supplement, and drug intakes were assessed.

Patients with history of diabetes, cancer, endocrinology disease, acute infection, use of special medications such as glucocorticoid, hormones, GnRH analogs, anticonvulsive drugs, heparin, aluminum containing antacids, thyroid hormones, lithium, chronic use of antiepileptic, omega 3 fatty acids, or other nutrients supplement, and smoking or alcohol consumption were excluded.

Study design

Forty-three patients in the treatment group and 39 patients in the control group were randomized by using Permuted Balanced Block Randomization Method. All participants received 1000 mg calcium and 400 IU vitamin D daily. The patients received two MorDHA capsules (435 g of DHA and 65 mg of EPA per day) or two placebo capsules (one with lunch, and another one with dinner) in the treatment and control groups, respectively. These were supplied in the form of gelatin pearls a day, taken with the two principal meals.

We used pill count method for assessment of compliance based on a confidence relationship between patient and physician and was reported at every 4 weeks. All participants recommended having a usual diet. Patients were not given specific advice on food intake during the study. No lifestyle, diet, or medication changes were recorded at every 4 weeks by phone call or face-to-face interview.

Calcium capsules were provided by Darou Pakhsh Pharm Co. (Iran). Omega 3 placebo capsules were provided by Minami Nutrition Co. (Belgium) and placebo capsules were supplied by Zahravi Pharmaceutical Co. (Iran). Both capsules were similar in color, shape, and taste. The patients were supplemented for 4 months.

Laboratory measurements

Antecubital venous blood samples were taken under antiseptic conditions in the post-absorptive state after an overnight (12 hours) fast. Blood samples were collected and centrifuged at 3 000 rpm for 10 minutes at 4°C. To reduce inter-assay variation in serum samples, single session analysis was used. Then, samples were sent to the Endocrinology and Metabolism Research Center (EMRC) laboratory for analysis and were frozen immediately. Serum TNF-α, IL-6, and IL-1β levels were measured using photometric enzyme-linked immunosorbent assay (ELISA) obtained from ID Labs (Canada) in the EMRC laboratory. The limit of detection serum for TNF-α, IL-6, and IL-1β were 8, 10, and 4 pg/ml, respectively.

Dietary intake

Dietary intakes of the patients were assessed by 3-d food records in the beginning and end of the study. The intake of calorie and the other nutrients was analyzed by using Nutritionist IV software (version 3.5.3.; N-Squared Computing, Salem, OR), modified for Iranian foods.[12,16]

Anthropometric measures

All measurements were conducted by the same investigator. Body weight was measured using a digital wheelchair scale, Body height was obtained measuring the supine length, and BMI was calculated using the formula: BMI (kg/m^2) = Body Weight (kg)/ (Body Height)^2 (m^2).

Neurologic assessment

SCI history included date of onset, cause of SCI, level and extent of SCI (complete *vs* incomplete). American Spinal Injury Association (ASIA) Impairment Scale was used

for the assessment of spinal injury in our participants.[17] Completeness was classified as either complete (no sensory or motor function preserved in the sacral segments [S4-S5]) or incomplete (sensory but variable motor function preserved below the neurological level of injury).[18]

Statistical analyses All statistical analyses were performed using SPSS software, Version 18.0 (SPSS Inc., Chicago IL, USA). Continuous variables are expressed as mean ± SD and categorical data are expressed as percentage. $P<0.05$ was considered as statistically significant. The distribution of continuous variables was assessed by Kolmogorov–Smirnov test. Baseline factors were compared between groups by Student's *t* test for continuous variables and by *Chi square test* for categorical variable. To investigate whether one of two groups changed more than another group at the end of 4th month, at first we calculated change scores (mean difference) for each group and compared them by using Student's *t* test.

RESULT

Patient characteristics

Forty-three patients were randomly assigned to the MorDHA group and 39 to the control group. Seventy-five (13 females, 62 males) patients completed the study over 4 months [Figure 1]. Four patients were dropout in the supplement group (one because he had spinal cord surgery and one because he lived too far away from the study site and two because of gastrointestinal adverse events that disappeared after stopping supplement intake). Three in the control group were dropout (one because he lived far away from the study site and two gastrointestinal adverse events that disappeared after stopping supplement intake).

There were no significant differences in age, BMI, time since injury between two groups [Table 1], and dietary intakes [Table 2]. Compliance with supplement averaged 80% in both groups over the 4 months of observation.

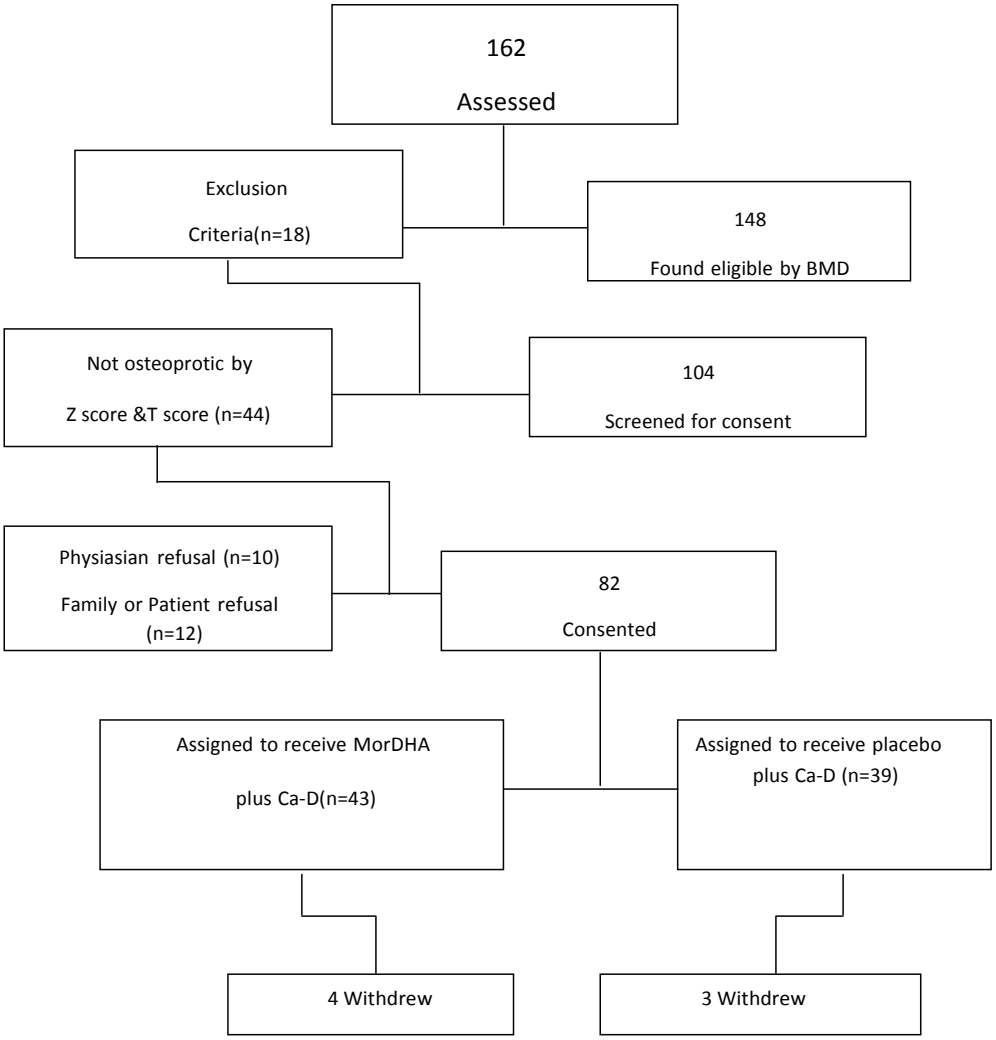

Figure 1: Study design of our study

Table 3 presents the between-group comparisons of the IL-1β, IL-6, and TNF-α as well as mean difference of all variables from the beginning to the end of the study. All variables in both groups have been improved to some extent but the difference between two groups was not significant in any.

Two adverse events occurred in the MorDHA group: (one diarrhea and one pyrosis); two gastrointestinal adverse events occurred in the control group. No hospitalization or death caused by adverse events occurred in the supplemented group.

Table 1: Baseline characteristics of participants

	MorDHA plus Ca-D (n = 39) Mean (SD)	Ca-D (n = 36 Mean (SD)	*P* (*t*-test)
Age (years)	40.11 (14.55)	38.36 (12.28)	0.3
Body mass index (kg/cm)	19.57 (4.09)	19.80 (3.57)	0.5
Time since SCI (years)	13.67 (25.87)	9.22 (7.49)	0.2
Neck.T	- 2.35 (1.23)	-2.41 (1.08)	0.83
Troch.T	-2.13 (0.8)	-2.04 (1.12)	0.70
Inter.T	-2.39 (0.95)	-2.52 (1.11)	0.60
Total.T	-2.46 (0.83)	-2.51 (1.14)	0.87
Z score (Neck.T)	-0.4 (0.93)	-0.45 (0.82)	0.83
Z score (Total.T)	-0.45 (0.78)	-0.48 (1.07)	0.87

No significant differences between two groups by *t* test

Table 2: Dietary characteristics of participants

	MorDHA plus Ca-D (n = 39) Mean (SD)	Ca-D (n = 36) Mean (SD)	*P* (*t*-test)
Calcium intake (mg/day)	816.52 (570.17)	786.32 (862.59)	0.9
Fat (gr/day)	85.8 (72.36)	56.68 (37.38)	0.3
Magnesium (mg/day)	303.93 (258.60)	1315.87 (567.27)	0.8
manganese (mg/day)	3.13 (2.28)	3.29 (5.89)	0.9
zinc (mg/day)	10.31 (5.71)	9.01 (6.87)	0.6
Kilocalorie Kcal/day	2002.52 (658.04)	1588.96 (709.24)	0.43
carbohydrate (g/day)	250.17 (91.63)	221.44 (93.78)	0.65
Cholesterol (mg/day)	321.30 (241.00)	245.51 (267.38)	0.21
Phosphorus (mg/day)	1285.03 (721.79)	1020.46 (690.02)	0.31
Copper (mg/day)	1.82 (3.38)	1.23 (1.34)	0.45
Selenium (µg/day)	.09 (.04)	.08 (.05)	0.34
Molybdenum (mg/day)	30.21 (38.48)	56.34 (171.98)	0.45
Fluoride (g/day)	18.9 (15.24)	14.43 (11.46)	0.8
Vitamin k (mg/day)	115.02 (121.24)	381.54 (1609.94)	0.23
Vitamin D (IU)	1.19 (1.63)	1.69 (3.03)	0.1
Dietary fiber (g/day)	13.02	13.97 (15.28)	0.3

No significant differences between two groups by *t* test

DISCUSSION

In vivo and *in vitro* studies have shown that the dietary n3-PUFA may play an important role in suppressing the production of bone absorption, pro-inflammatory cytokine, and improving of lipid profile.[5,19-27] However, data are very few in human studies.[9,28] Until now, no conclusive data are available in SCI patients.

This study evaluated the anti-inflammatory aspect of omega-3 fatty acids relative to cytokines and their clinical effects in osteoporotic SCI patients. Mean differences in all pro-inflammatory cytokines were not significant in both treatment and control groups during the study ($P>0.05$).

In some recent studies, n-3 PUFA present in fish oil that were shown to decrease the production of three inflammatory cytokines, IL-6, IL-1 β, and TNF-α *in vitro*.[5,9,28,29] Recent studies in normal volunteers indicate that the beneficial effects of dietary PUFA on risk of chronic inflammatory disease (cardiovascular disease, rheumatoid arthritis, asthma, and periodontitis)[5,10] is based on decrease in production of interleukins.[9]

It seems that the anti-inflammatory effects of DHA may be much more effective than EPA[6-8,30] It might be, in part, due to the inhibition of transcription nuclear factor-κB intracellular signaling activation and activation of another transcription factor peroxisome proliferator-activated receptor-γ.[6,7,9,31] In a recent study, treatment with fish oil for two years retards the rate at which renal function is lost.[32] The omega-3 fatty acids in fish oil affect cytokine production in patients with IgA nephropathy[32] and lupus nephritis.[33,34] Kelley *et al*.[35] evaluated the effect of DHA on the production of TNF-α and IL-1 in young healthy men. They were shown that DHA (6 g/d) had no effect on cytokines production.

In agreement with our study, some human studies had shown no effect of fish oils on pro-inflammatory cytokines.[28,36] In another double-blind study, 42 healthy subjects were randomly allocated to receive supplementation with either

Table 3: Between group differences in Serum IL-1β, IL-6, and TNF-α of variables from the beginning to the end of the study {Mean (SD)}

		MorDHA group*			Control group**	
Parameter	Mean difference	0 months	4 months	Mean Difference	0 months	4 months
Il1-ß (pg/ml)	-23.70 (24.60)	46.47 (18.42)	31.54 (46.33)	-22.01 (28.79)	46.75 (14.06)	27.71 (32.42)
IL6 (pg/ml)	-16.54 (38.41)	66.46 (36.25)	49.92 (26.51)	-11.63 (31.94)	62.48 (25.86)	50.48 (23.82)
TNF-α (pg/ml)	1.16 (23.12)	52.88 (64.96)	35.84 (19.66)	4.59 (16.52)	34.91 (17.94)	30.32 (9.09)

*MorDHA group: 1000mg (435 mg DHA, 65 mg EPA) (Minami Nutrition Co. Belgium) plus Ca (mg1000)- Vitamin D (400 IU) (Darou Pakhsh Drug Co. Iran); **Placebo: Gelatin 1000 mg (Zahravi Pharmaceutical Co. Iran) plus Ca (mg 1000)- Vitamin D (400 IU) (Darou Pakhsh Drug Co. Iran); - No significant differences between two groups by *t* test

placebo (olive oil), EPA (4.7 g/d), or DHA (4.9 g/d) for 4 weeks.

No significant effect of fish oils was found on cytokine production.[28] In a study,[37] researchers evaluated the effect of n-3 fatty acids on bone biomarkers in 25 osteoporotic post-menopausal women. Patients received 900 mg n-3 fatty acid capsules or placebo for 6 months.

No significant changes were seen in bone resorption or formation markers, except for urine pyridinoline in this study.

Given the continued interest in the modulatory effects of n-3PUFAs on inflammation-based bone resorption and lack of information about SCI patients, led ours to design this study to investigate the effect of omega-3 fatty acids on inflammatory cytokines in SCI patients. However, in most of these studies, the dosage and period of supplementation were substantially greater than those used in our study.

It seems that considerable inconsistency has been shown in the reported effects of n -3PUFAs on production of inflammatory cytokines. This variation may be due to differences in doses that are used in variable studies.[28,36] However, some studies using high doses of n -3 PUFAs[28,38-40] showed no significant positive effect, whereas others using low doses have shown inhibitory effect on production of cytokines.[40] It seems that these researches may estimate their power studies based on earlier studies that evaluate the effect of n3-PUFAs on inflammatory cytokines and bone modulation with flawed design. Consequently, the later studies failed to confirm the results of the earlier studies. Therefore, reevaluation of this interesting field with responsible design, sample size, and different doses is clearly essential.

LIMITATIONS

In spite of the fact that both genders were used in our study along with a greater number of participants, our sample was still fairly small and limited our power to recognize small differences between groups. Moreover, time scarcity was another limitation of our study. Our study was done in 4 months, and it seems that estimation of BMD can give us more reliability. So, designing of a study with a longer duration of at least 1 to 2 years to evaluate the changes of BMD in parallel with inflammation markers could be helpful.

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