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The Combined Effects of Omega3 Fatty Acids and Nano-Curcumin Supplementation on Gene Expression and Serum Levels of Some Inflammatory and Endothelial Factors in Migraine Patients: Studyprotocol for a Randomized Controlled Trial

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ABSTRACT: *Migraine is a chronic nerves system disease leads to considerable disabilities and affected quality of life. Neuro-inflammation play a key role in progression of migraine which mainly caused by release of inflammatory mediators including COX-2 and iNOs enzymes, TNF-α،IL-1β،IL-6 and adhesion molecule such as ICAM and VCAM.Curcumin and omega 3 fatty acids with anti-inflammatory and neuro-protective effects can reduce gene expression and production of inflammatory mediators. The combination of omega-3 and curcumin have also synergistic effects which a lower dose can induce a positive effect. The aim of present study is to determine whether omega 3 fatty acids, curcumin or combined of them as a complement treatment in migraine is helpful and the reduction gene expression and serum levels of inflammatory markers.The study will be conducted involving 80 episodic migraine patients that withstratified randomization method based on sex, gender and body mass index (BMI) are classified into 4 groups: 1) receiving omega 3 fatty acid supplement (1.8 gr/ day) and nano-curcumin supplement (80 mg/ day) 2) receiving omega 3 fatty acid supplement and curcumin placebo 3) receiving curcumin supplement and omega 3 fatty acid placebo 4) receiving omega 3 fatty acid placebo and curcumin placebo for 2 mounts. Blood samples will be collected after anthropometric parameter measuring then target biochemical parameters, gene expression and serum levels oftarget inflammatory mediators including COX-2 and iNOs enzymes, TNF-α،IL-1β،IL-6 and adhesion molecule such as ICAM and VCAM will be measured before and after the trial. The data will be statistically evaluated using the most appropriate tests.The results of current study will determine the efficacy of therapeutic effects of curcumin, omega 3 fatty acids and combined of them as a new insight to control and treatment of migraine.*

Keywords: *migraine,nano-curcumin, omega 3 fatty acid, gene expression*

I. Introduction

Migraine is a common and chronic neuroinflammatory disease with progressive and episodic nature leads to considerable disabilities and affected patient's activity, psychological status and quality of life. It also exert a heavy economic burden to society and person [1, 2]. Pathogenesisof migraine is not clear and there is not decisivetreatment for migraine already [3]. The epidemiological studies suggested that prevalence of migraine isincreased from 6% to 13% in all over the world during 5 years. In United States, also migraine's prevalence increased about twofold in recent decade and included 23.5% of population. One of 4 persons and one third of women suffered from degree of migraine in US [2, 4, 5]. Migraine involved about 6-8% of men and 15-20% of women in eastern countries. The elevated prevalence of migraine cause that it classified as 20 disability-related conditions and also as 10 most prevalent incapacitating diseases [6, 7].

The importance of migraine, not only because of its suffering disruptive pain, but also is due to inflammatory and progressive nature and its usual related disease such as depression, anxiety, hypertension, stroke and cardiovascular disease [8, 9].

Many factors are proposed in pathogenesis of migraineincluding genetics factors, elevated levels of glutamate between and within the attack phases, magnesium deficient, monoaminergic pathway disorders (serotonin and dopamine), mitochondrial dysfunctions, elevated calcitonin gene-related peptide (CGRP) and neurogenic inflammation[10].During the active phase of the disease, neuronal activities has increased and leading to the release of pro-inflammatory peptides from neuronal terminals of around vessels resultedin increasing oxidative stress, activation of leukocytes and adhesion molecules, inflammation and finally dilation of vessels in and out of the skull. Subsequently repeated vascular inflammation causes endothelial damage and atheropathy. Several studies confirmed the role of inflammation in the development and progression of migraine [11, 12]. Neuro-inflammation in migraine is mainly caused by activation of sensory neurons and then release of inflammatory mediators such as CGRP. This activation increased the activity of inducible Nitric oxide synthase (iNOs) and elevated nitric oxide (NO) production and also induced the gene expression and activity of cyclohexane oxygenase -2 (COX-2) enzymes that results in release of inflammatory cytokines including interleukin (IL)-6, IL-1β and tumor necrosis factor (TNF)-α. Subsequently this inflammatory phenomenon exacerbated nociceptive activity and sensitivity [13].Cytokines play an important role in pain, inflammation and pathogenesis of migraine through increasing the membrane permeability and cell-to-cell interactions. Cytokines and its receptors are widely expressed in neurons and central nervous system. It suggested that the injection of TNF-α and IL-6 in central or peripheral vessels could cause hyperanalgia. Several studies also demonstrated that in migraine's patients the concentration of pro-inflammatory cytokine such as IL-1, IL-6 and TNF-α were increased that result in vascular dysfunction [14]. In patients with migraine, vascular dysfunction is also associated with activation of vascular endothelial and increase in production of pro-inflammatory cytokine and adhesion molecule (includingintercellular adhesion molecule (ICAM) and vascular cell adhesion molecule (VCAM)). Inflammatory cytokine such as TNF-α have a vasodilator properties and induced the expression of ICAM and VCAM, so that elevated expression of this factors associated with activation of microglia which causes inflammation, neuropathic pains and subsequently brain inflammation [2, 15 , 12].

There is some medication to treatment of migraine including Non-steroidal anti-inflammatory drugs (NSAIDs) and COX-2 inhibitor which suppressed inflammatory process [14]. Another drugs that used for migraine is valproate, and its mechanism included inhibition of inflammatory cytokine's mRNA expression such asTNF-α, IL-1β, IL-6andIL-17 and also through inhibition of macrophages and B, T cells aggregation which leads to suppression of inflammation [16].

Therefore, the Therapeutic and nutritional approach that leads to inhibition or reduction of releasing inflammatory cytokines included TNF-α ،IL-1β ،IL-6 or result in improve endothelial function with decrease in expression of endothelial dysfunction markers (ICAMandVCAM) and also could inhibited the COX-2andiNOs enzymes, will be effective in healing and preventing progression of disease. Several in vitro, in vivo and clinical trial studies in neuro-inflammatory and neuro-degenerative fields suggested the similar mechanisms for curcumin (the active substance of Turmeric) and omega-3 fatty acids with migraine's medication.

Curcumin is a lipophilic substance that remains intact in the acidic PH. Curcumin has a several antioxidant and anti-inflammatory properties from influence on gene expression to cellular signaling. Curcumin suppressed secretion of cytokines including TNF-α ،IL-1 ،IL-6 ،IL-8and IL-12 and also is able to inhibit COX-2andiNOs enzymes in various types of cells. In addition, curcumin inhibits NF-κB and suppresses expression of ICAMandVCAM [17].

Nuclear factor-κB (NF-κB) is an axial factor that has an important role in upregulation of inflammatory cytokine, adhesion molecule and cox-2 gene expression. Curcumin with its inhibitory effect on gene expression and NF-κB activity has a considerable role in reducing the inflammation.The in vivo studies also demonstrated that curcumin could alleviate neurogenic pains through reducing TNF-α ،IL-1β ،NOand NF-κB gene expression and with effect on spinal monoaminergic systems [18]. Beneficial effects and neuro-protective properties of curcumin in disease with neuro-inflammatory basis including Alzheimer, multiple sclerosis, Parkinson and etc., have been approved in several animal and human studies [19]. Curcumin has a synergistic effect in enhancing anti-inflammatory and pain relieving with NSAIDs [20]. Therefore, it can be used in treatment of inflammatory disease as an alternative therapy or decreasing the medication' doses.

In addition to curcumin, a range of in vitro and in vivo studies confirmed the anti-inflammatory and neuro-protective effects of omega-3 fatty acids. Although the exact mechanisms of biological functions of omega-3 fatty acids was not fully understood, but previous studies demonstrated that the beneficial effects of docosahexaenoic acid(DHA) and eicosapentaenoic acid (EPA) in brain and nervous system take place through reducing the production of inflammatory cytokines and their influences on vasodilatation (by inhibiting the production of NO) [21]. Omega 3 fatty acids have an anti-inflammatory effects through multiple mechanisms. EPA and DHA as PPAR-ϒ ligands by inhibiting NF-κB signaling could suppressed the production of inflammatory factors and cytokine including TNF-α ،IL-1β ،IL-6 and enzymes activity such as COX-2andiNOs. In adition, their derivate such as resolvins also play an important role in reducing the production of inflammatory cytokines in neurons. Previous studies suggested that rich sources of long chain fatty acids such as

fish oil may be able to improve vascular functions and decrease production of vascular dysfunction markers including ICAM and VCAM [22, 23]. Omega-3 fatty acids with similar mechanism to COX-2 inhibitory drugs have beneficial effects in reducing inflammatory and neurogenic pain through decreasing in pain receptor's expression and activity. EPA and DHA not only have not the side effects of this drugs, but also have an neuro-protective properties and can prevent from neurologic, inflammatory and vascular disease [24].

It has been demonstrate that omega-3 fatty acids have synergistic effects with NSAIDs and valproate which use in migraine [25], on the other hand, curcumin also enhance the NSAIDs effectswith its synergistic effect [20].The combination of omega-3 and curcumin have also synergistic effects which a lower dose can induce a positive effect [26]. Both of these compounds, in addition to their beneficial effects on reduce inflammation and cardiovascular function, have a neuroprotective properties, so that combined effects of these compounds can be targeted as a drug for treatment of neuroinflammatory diseases such as migraine and even could be replace with their medication.

The objective of present study is to determine the effects of omega 3 fatty acid, curcumin and their combination or placebo for 2 months on the gene expression of COX-2, iNOS, TNF-α, IL-1β, IL-6, VCAM-1 and ICAM-1 in the peripheral blood mononuclear cell (PBMC) and serum levels of COX-2, iNOS, TNF-α, IL-1β, IL-6, VCAM-1, ICAM-1 and high-sensitivity C-reactive protein (hsCRP) of migraine patients.

II. Methods/ Design

Randomized double blind placebo controlled trial will be conducted involving 80 patients with migraine recruited from the Iranian Center of Neurological Research. the study was approved by Ethics Committee of the Tehran university Medical of Sciences (TUMS) (Number 28825) on July 2015 and it is identified in ClinicalTrials.gov as ID: NCT02532023.

2.1. Inclusion criteria

The inclusion criteria including patients with episodic migraine recognized according to International Headache Society (IHS) criteriaby neurologist at the age range of 20 to 50 years old; BMI mor than 18.5; avoidance of any dietary supplements andvitamins at least 4-6 weeks before and throughout the intervention; don't suffering from kidney disease, liver damage, pancreatitis, diabetes, cancer, thyroid disorders and inflammatory diseases or history of heart disease and stroke according to patients statement and their medical history; normal blood biochemical tests before the start of study.

2.2. Exclusion criteria

The exclusion criteria including allergic reaction to the components of omega-3 and curcumin; patients who are unwillingness for cooperation; pregnancy and lacation; any drastic change in diet and regular life style; any change in type and dosage of regular medication (s) (Nortriptyline and Inderal); patients with inflammatory disease that leads to take long-term anti-inflammatory drugs (more than 2 weeks) and consumption alcohol and smoking (at least 5 cigarettes per day during the last 6 months).

2.3. Study design and experimental groups

participants who meet the eligibility criteria and be agree to enroll to study will sign a statement of informed consent approved by Ethics Committee of the TUMS. Selected samples by using stratified randomization method based on sex, gender and body mass index (BMI) are classified into 4 groups: 1) The group receiving supplements of omega-3 (two capsules per day, each capsule containing EPA: 600 mg and DHA: 300 mg) and curcumin placebo, 2) the group receiving supplements of nano-curcumin (one capsules per day, each capsule containing 80 mg) and omega3 placebo, 3) The group receiving a combination of omega-3 supplements and curcumin 4) and control group that will receive omega3 and curcumin placebo for two months. The omega 3 fatty acid placebo group will also receive placebo containing 1800 mg edible paraffin oil and curcumin placebo group will receive 80 mg starch powder. Placebo and supplements are similar in terms of color, shape and size. Gelatin capsule supplements and placebo will prepare in thirty packages, each for one month consumption and stored at room temperature. Supplements will be delivered to the Patients in two stages (to use for a month). To keep informed of regular intake of supplements and possible problems during the study, once every two weeks calls with patient and also on the second visit, ask them about how to consume supplements. Patients are asked to return boxes of drugs. Consumption less than 90% of the supplement at the end of two months will be considered as non-compliant. participants are advised to maintain their diet, level of physical activity and medication dose during the study. Blood samples will be collected after anthropometric parameter measuring then target biochemical parameters, gene expression and serum levels and physical activity will be measured before and after the trial.

2.4. Questionares

At the start of the study a set of questionnaires includinggeneral informationquestionnaire, 24-hour dietary recall questionnaire and physical activity questionnaire will be completed for every patients by a trained subject. For filling general information questionnaire, the participants will be asked about age, education, job, smoking and alchohol consumption, medical history includes a history of diseases such as diabete mellitus, renal and liver disorder, myocardial infarction, strok, asthma, allergy, cancer, immune disease and so on as well as history of drug consumption, any dietary supplements and vitamin/ minerals.Also headache severity, duration and the naumber of attack in patientsat the bigining and at the end of study will be recorded.

For nutritional assessment three 24-hour dietary recall questionnaire at the beginning and end of the study consists of two typical day and a holiday is taken.

For evaluation of The physical activity level the short form of physical activity questionnaire (IPAQ) will be used. participants are advised toreport time spent on moderate or vigorous intensity, walking and sitting down during the week prior to test[27].

2.5. Anthropometric measurement

anthropometric parameters including weight, height and waist circumference are measured. The weight of patients is measured with minimal clothing and without shoes by digital scale (Clara 803) to the nearest 0.01 gram. Height will measure using a wall stadiometer in standing position without shoes with a precision of 0.1 cm (seca, Germany). Waist circumference will be measured in the middle of lower rib margin and iliac crestby tape (seca 201model) in standing position and breathing normally.

2.6. Sample blood collection and PBMC separation

Blood samples (15- 20 cc) will collectedfrom participants in sterile tubes with heparin as ananticoagulant.Two ml of blood are poured in CBC tubesfor counting blood indexes including RBC, Plt, Hb, Hct, MCV, MCH and MCHC. For serum separation samples are centrifuged at 3000 RPM for 10 minutes then one ml of whole blood samples wascollected in a sterile microtube without anyanticoagulantand store at -80 ° C until the serum markers of the study measured. PBMCs wereisolated using the Ficoll-Histoprep gradient (BAGHealth Care GmbH, Germany) centrifugation protocol [28].

2.7. Determination of inflammatory markersserum Levels

Serum levels of inflammatory marker will be measured in separated serum obtained from patients**.** For this purpose the serum activity of COX-2 and iNOS enzymes, serum levels of pro-inflammatory cytokine comprise TNF-α, IL-1β and IL-6, and endotehelial factors including soluble VCAM-1 and ICAM-1 were measured by ELISA kit according protocol of company (eBioscience USA) in all of group of study before and after intervation.

2.8. Determination of inflammatory markersgene expression

peripheral blood mononuclear cells are isolated using standard protocols then RNA was synthesized from cells by RNeasy Mini Kit (Qiagene-USA). After this, cDNA was synthesized from RNA by a QuantiTect Rev (Qiagene-USA) and the gene expression of inflamatory enzymes, endothelial factors and cytokines including COX-2 and iNOS enzymes, TNF-α, IL-1β and IL-6 and VCAM-1 and ICAM-1will be quantified by RT-PCR at the bigining and at the end of study.

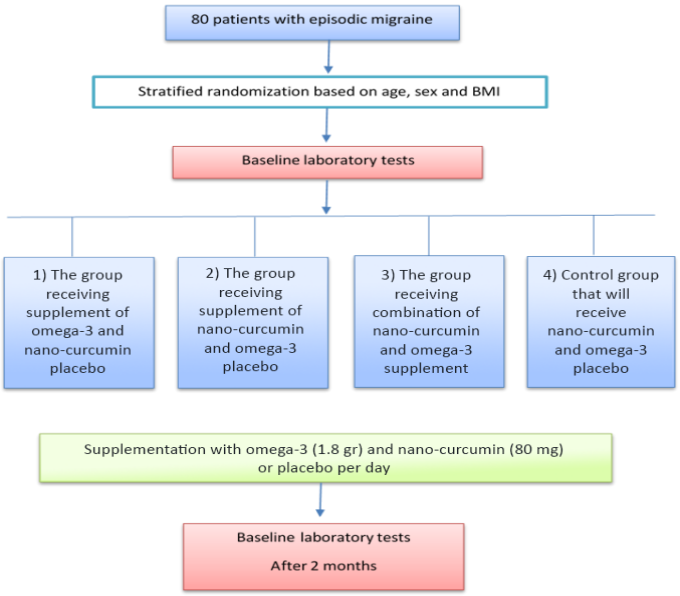

Figure 1. A summary of the interventional study

III. Data Analysis

Data are expressed as means ± SD. Data analysis is performed using SPSS software version 21. For compression between before and after variables and all study groups toghether paired t test and ANOVA test will be used respectively. Linear regression model use to investigate the association between quantitative variables. If two variables were linearly related, PEARSON correlation coefficient is use and if pairwise comparison was considered, SPEARMAN test is used. As well as to eliminate the effect of confounding variables used ANCOVA test.The test level for statistical significance of differences between four treatment arms is defined as $p \leq 0.05$ for all tests.

IV. Conclusion

On the basis on evidences curcumin and poly unsaturated fatty acids with similar mechanism to anti-inflammatory drugs have an important role in determining the severity of inflammatory disease, vascular disorders and reducing neurogenic pain.It is possible dietary supplemention of patients with inflammatory and vascular disease with curcumin and omega-3 fatty acids have clinically usefull effects on relieving thedisease. Also the beneficial effect of this compounds in neuroinflammatory and neurodegenerative disease have been approved in animal and human studies. Its demonstrated that Omega-3 fatty acids and curcumin have a synergistic effects with drugs used in migraine. The studies also suggested that curcumin and omega-3 fatty acids have a synergistic effects and are able to boost the effects of each other. Therefore, this compound can be targeted to reduce the dose of drugs used in migraine or even be replaced to medication. on the other hand, there is no studies that assess the effects of omega-3 fatty acids, curcumin and their combined effects on gene expression of infammatory cytokine, enzymes and vascular factors which have an considerable role in migraine's pathogenesis and current study for the first time is designed toclarify the exact mechanism of this compound and mediated pathway in migraine's pathogenesis.

Abbreviations

CGRP: Calcitonin Gene-Related Peptide; COX-2: Cyclohexane Oxygenase -2; DHA: Docosahexaenoic Acid; EPA: Eicosapentaenoic Acid; hsCRP: high-sensitivity C-Reactive Protein; ICAM: Intercellular Adhesion Molecule; Hb: Hemoglobin; Hct: Hematocrit; HIS: International Headache Society; IL: Interleukin; iNOs: inducible Nitric Oxide Synthase; MCH: Mean Corpuscular Hemoglobin; MCHC: Mean Corpuscular Hemoglobin Concentration; MCV: Mean Corpuscular Volume; NO: Nitric Oxide; NSAIDs: Non-Steroidal Anti-inflammatory Drugs; PBMC: Peripheral Blood Mononuclear Cell; Plt: platelet; RBC: Red Blood Cell; TUMS: Tehran University Medical of Sciences; TNF: Tumor Necrosis Factor; VCAM: Vascular Cell Adhesion Molecule.

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