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Evaluation of the Effectiveness of Ellagic Acid on Liver Steatosis in Overweight/Obese Non-Alcoholic Fatty Liver Disease Patients: Study Protocol for a Randomized Controlled Trial

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ABSTRACT

Introduction: Non-alcoholic fatty liver (NAFLD) is a condition that can lead to liver fibrosis, cirrhosis, and hepatocellular carcinoma. There is no definite treatment for NAFLD. Ellagic acid (EA) is natural polyphenol with proven pharmacological properties, but its effectiveness on NAFLD in patients is not clear. The aim of this study is to evaluate the effect of ellagic acid on liver fibrosis in NAFLD patients.

Method: This randomized, double-blind, controlled, and parallel clinical trial will be conducted on 60 patients with NAFLD (grade 2 or above) who will be allocated in intervention or placebo groups with 1:1 ratio. The intervention group will receive capsules containing 200 mg EA while the placebo group will receive avicel as placebo for 8 weeks. Body composition and anthropometric indices will be measured at baseline, end of 4th and 8th week. Transient elastography, 3-day food record, International Physical Activity Questionnaire (IPAQ), and laboratory tests (CBC/diff, uric acid, creatinine, lipid profile (total cholesterol, triglyceride [TG], low-density lipoprotein cholesterol [LDL-c], and high-density lipoprotein cholesterol [HDL-c]), aspartate aminotransferase [AST], alanine aminotransferase [ALT], alkaline phosphatase [ALP], and gamma-glutamyl transpeptidase [GGT], albumin, high sensitive C-reactive protein [hs-CRP] and insulin) will be performed for both groups at baseline and end of the study.

Conclusion: We expect that 200 mg EA ellagic acid administration for 8 weeks will improve liver steatosis, clinical and paraclinical outcomes in NAFLD patients.

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Abbreviations

ALP: Alkaline Phosphatase
ALT: alanine aminotransferase
AST: aspartate aminotransferase
BMI: Body Mass Index
CBC-diff: Complete Blood Count with Differential Count
hs-CRP: High-sensitivity-C reactive protein
EA: ellagic acid
FBS: Fasting Blood Sugar
HbA1C: Hemoglobin A1C
HDL-c: high density lipoprotein-cholesterol
IPAQ: International Physical Activity Questionnaire
LDL-c: low density lipoprotein-cholesterol
NAFLD: Non-alcoholic Fatty Liver Disease
NASH: non-alcoholic steatohepatitis
RCT: Randomized Clinical Trial
GGT: Gamma-glutamyl Transferase
TG: Triglycerides
TC: Total cholesterol

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Introduction

Non-alcoholic fatty liver (NAFLD) is a pathological liver condition characterized by accumulation of fat in hepatocytes, in the absence of alcohol abuse (more than 30 gr/d for men, more than 20 gr/d for women) or other liver diseases, including hepatitis or metabolic liver disease [1]. NAFLD is a progressive disease which can progress to non-steatohepatitis (NASH), liver fibrosis, and finally cirrhosis or hepatocellular carcinoma [2]. NAFLD is found in more than 25% of adults in the world [3]. NAFLD prevalence in Iran is 33.9 % [4]. There is a close relationship between NAFLD and other metabolic conditions such as type 2 diabetes mellitus, cardiovascular disease, metabolic syndrome, insulin resistance, dyslipidemia, and obesity [5, 6]. The only gold standard for NAFLD diagnosis is liver biopsy.

Liver biopsy is invasive and has many complications, including haemobilia, pain, intraperitoneal bleeding, and rarely death (rate: 0.009% to 0.14%) [7-11]. Two-dimensional elastography is a non-invasive method with acceptable diagnostic performance that is able to quantify the extent of fibrosis and steatosis [12]. The advantages of elastography over liver biopsy include being fast and painless with no bleeding and complications have made it an acceptable diagnostic method for patients [13].

Today, the "multiple-hit theory" is described as the interaction between different factors in the development of NAFLD. Based on the multiple-hit theory, increased plasma level of free fatty acids, liver inflammation, insulin resistance (IR), hormones secreted by adipocytes, oxidative stress, intestinal microflora, nutritional, genetical and epigenetic factors are involved in NAFLD. It seems that fat accumulation and insulin resistance are the first hit. Some intestinal bacterial products, including lipopolysaccharides, small chain fatty acids, and bile acid metabolic products were linked to liver inflammation and NAFLD, however; causation is not determined [1]. Recent epidemiological observations showed that patient with NAFLD have increased serum level of uric acid [14, 15]. More studies showed that high level of serum uric acid can exacerbate NAFLD. Possible mechanisms for NAFLD include mitochondrial oxidative stress, activation of nucleotide oligomerization domain receptor protein, and reduced IR [16].

No proven treatment exists for NAFLD [17]. High fat diet is linked to liver steatosis [18]. Lifestyle modification including physical activity and maintaining a healthy diet is the best preventive strategies for NAFLD and its progression [19-21]. Modification of the amount of fat intake and type of fat can affect the fat metabolism in hepatocytes [22].

Ellagic acid (EA) is a natural polyphenol compound found in many fruits, including peach, pomegranate, grape, strawberry, raspberry, plum, green tea, and some nuts (walnut and almonds). Studies have approved the safety of EA and it is approved as a food additive by countries like Japan [23]. EA structure contains 4 phenolic groups and 2 lactones groups which take part in redox reaction and inhibition of several nitrogen and oxygen reactive species [24-28]. Hydrogen ion (H^+) released from phenolic group neutralizes free radical species [29]. Various EA derivative compounds like its glycoside and methylated forms are found in vegetables. Intestinal microflora converts these derivatives to urolithins (UTs) [30, 31]. EA has properties, including anti-inflammatory, anti-viral, anti-oxidant, anti-metastatic, anti-hyperlipidemic and anti-cancer properties [32]. To the best of our knowledge, the effects of EA on steatosis, liver stiffens, liver enzymes and some inflammatory markers has not been fully studied. Therefore, this study aims to evaluate of the effectiveness of EA administration on liver steatosis in patients with NAFLD.

Study Objectives

Primary objective

To measure the difference in steatosis before and after the intervention in treatment and control groups, and to compare the within and between group changes

Secondary Objective

1. To compare fibrosis changes during the study period between the treatment and control groups
2. To compare changes in TC, TG, LDL-c, HDL-c during the study period between the treatment and control groups
3. To compare changes in ALT, AST, ALP, GGT, albumin, FBS, IL-6, hs-CRP, fasting insulin, uric acid and creatinine during the study period between the treatment and control groups
4. To compare changes in HOMA-IR index during the study period between the treatment and control groups
5. To compare changes in energy, carbohydrate, protein and fat intake during the study period between the intervention and control groups

6. To compare changes in anthropometric indicators (weight, height, BMI, fat free mas, fat mass and waist circumference) during the study period between the intervention and control groups
7. To determine IPAQ score at baseline and endpoint in intragroup and between groups
8. To determine incidence of probable adverse effect and patients' tolerance

Materials & Methods

Study Design and Setting

The present study is a randomized and double-blind phase II (2A) clinical trial study. The study has two parallel arms. The study protocol received ethical approval from the Research Ethics Committees of the Mashhad University of Medical Sciences (ethics number: IR.MUMS.REC.1400.223). Also, this study was registered in Iran's clinical trial website and the IRCT code was received with ID IRCT20180103038199N16. The study protocol is prepared based on Recommendations for Clinical Interventional Trials (SPIRIT) guidelines. Table 1 shows a brief view of the study protocol. Simple randomization will be used to allocate eligible participants to either the control or intervention groups.

Table 1. A SPIRIT diagram of the recommended content for the schedule of study

TIMEPOINT	Enrolment	Allocation	Intervention			Close-out
	0 to first 24h	0	Baseline	end of 4th week	end of 8th week	
ENROLMENT:						
Eligibility screen	*					
Informed consent	*					
Past medical history	*					
Allocation		*				
INTERVENTIONS:						
Ellagic acid			*	*	*	
Placebo			*	*	*	
ASSESSMENTS:						
Liver share wave elastography	*				*	
CBC/Diff			*		*	
FBS, HbA1C, insulin			*		*	
Lipid profile			*		*	
Urea, Cr			*		*	
Liver enzymes			*		*	
Inflammatory markers			*		*	
HOMA-IR			*		*	
demographic information			*			
Weight measurement			*	*	*	
3-day food recall			*	*	*	
Body composition			*	*	*	
IPAQ			*	*	*	
FOLLOW-UP						
Inquiring whether patients wish to continue with their current diet				*	*	*

Sample Size

Sample size was calculated based on the hepatic steatosis classification change reported in a previous study [33] using the equation for comparing two ratios considering type I and II errors of 5% and 20%, respectively. The sample size was 26 patients in each group, which was increased to 30 patients in each group considering 10% dropout rate.

Participants

Inclusion Criteria

1. 30-65 years old
2. BMI: 25-35 kg/m^2
3. Confirmed diagnosis of NAFLD with elastography (grade 2 and higher)
4. Liver fibrosis (only grade 0 and 1)

Exclusion Criteria

1. Refusal to continue the study
2. Occurrence of unwanted side effects, including hypersensitivity reactions

Non-Inclusion Criteria

1. Alcoholic fatty liver and consumption of alcoholic products (more than 30 g/d for men and more than 20 g/d for women)
2. Viral hepatitis (especially hepatitis C)

3. Consuming medications, including amiodarone, corticosteroids, methotrexate, tamoxifen, synthetic estrogens, valproic acid, intravenous tetracycline, highly active antiviral drugs, lithium, 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, acetaminophen, salicylate, phenytoin, benzodiazepines, perhexiline, aspirin, and contraceptives.
4. Documented Wilson's disease, hemochromatosis, celiac disease, cirrhosis, biliary obstruction and primary biliary cirrhosis
5. Special nutritional conditions, including starvation, fasting and intravenous feeding
6. Smoking
7. Pregnancy or lactation
8. History of hypersensitivity to pomegranate
9. History of breast cancer, sclerosing cholangitis, kidney failure, autoimmune and malignancy
10. Taking multivitamin-mineral and omega-3 supplements three months before entering the study
11. Diabetes
12. Taking any type of herbal supplements

Data Collection at the Outset

At baseline, data on liver steatosis, stiffness and fibrosis; and blood tests (complete blood count (CBC/Diff), glycemic status, liver enzymes, lipid profile and inflammatory markers. Also, relevant questionaries, International Physical Activity Questionnaire (IPAQ) and food record will be collected.

Randomizing and Blinding

Sequentially numbered, opaque, sealed envelopes (SNOSE) will be used for Allocation Concealment. In this way, the envelopes will be prepared by one of the team members and random numbers will be printed and placed inside the envelopes. The opaque envelopes will be sealed and its contents will not be visible from the outside. After explaining the purpose of the study to the patients who meet the inclusion criteria. The willing patients will be asked to sign a written informed consent and take an envelope. The patient will be allocated to either group based on the number in the envelopes. The intervention and placebo groups will be presented with letters A and B, respectively so that patients will be blinded to their allocation group. Allocation ratio will be 1:1 and six combinations of the blocks with the size of 4, including 1. AABB, 2. BBAA, 3. ABAB, 4. BABA, 5. ABBA, 6. BAAB, (permutation block sampling) will be used for allocation.

Considering the sample size (n=60), 15 blocks will be needed for allocation. Random numbers between 0 and 6 will then be generated using random number maker software:

6 2 5 4 3 5 5 6 5 1 3 2 5 5 3

In the next step, the 15 combinations corresponding to the numbers will be recorded as follows:

BAAB, BBAA, ABBA, BABA, ABAB, ABBA, ABBA, BAAB, ABBA, AABB, ABAB, BBAA, ABBA, ABBA, ABAB

Then, patients will draw one of the envelopes and receive the treatment based on the letter (A or B).

Procedures

I) Preparation of capsules

The capsules used in the intervention group will contain 200 mg EA and 50 mg avicel, while the placebo group will receive capsules containing 250 mg avicel. The capsules administered to both groups will be similar in appearance. Brown confectionery color will be used for uniformity of the color of the capsule content in both groups.

II) Intervention

Patients who visit The Gastroenterology Clinic of the Imam Reza Hospital and fulfil the inclusion criteria will be selected and will be briefed about the study. All patients are required to provide informed consent to the clinician before participating. Patients will be randomly assigned to intervention or placebo groups based on the method described in Randomizing and blinding section. Randomization will be based on simple block randomization method. Sixty patients will be allocated to the intervention and placebo groups equally (n=30 in each group, total=60). The intervention duration will be 8 weeks. After preparing the randomization list using the random block method with a block size of 4, people are randomly assigned to two intervention and placebo groups. At the beginning of the study, 30 capsules will be given to each patient. Patients will be asked to return to the clinic after end of 4th week for further evaluations and to receive another 30 capsules. To control the confounding effect of food intake, patients in both the intervention and placebo groups will receive a hypocaloric diet, consisting of 55% carbohydrates, 30% fat, and 15% protein. Additionally, patients will be encouraged to perform 150-300 min/week moderate-intensity

or 75–150 min/week of vigorous-intensity physical activity based on the world health organization recommendation [34]. The dose of EA was selected based on a previous study that indicated the ingestion of 200 mg/d EA for 8 weeks was safe [35]. Furthermore, the mentioned study showed improvement in glycemic status, insulin resistance and oxidative stress in patients with polycystic ovarian syndrome.

III) Follow up

At the end of every week, patients will be contacted and reminded of the importance of following the diet, physical activity, and regular intake of the capsules. The second and third in-person visits will be scheduled by researcher after 4th and 8th week, respectively.

Safety Considerations

A trained medical specialist will be involved in study to address side effect reports by the patients. Based on the specialist decision, patients who report side effects may receive treatment, referred to specialist, or be advised to schedule an appointment with their primary practitioner as necessary. Patients will receive clinical and biochemical assessments, including the evaluation of liver enzymes. Supplementation will be immediately ceased in case of dangerous side effects or based on the decision by the specialist.

Data Collection and Analysis

Patients will have 3- consecutive visits: at baseline, after 4th and 8th week. After giving an informed consent, demographic questionnaire will be filled at baseline. Elastography will be done at baseline and at the end of the 8th week. Body composition will be checked using bioimpedance analysis (BIA) device at baseline, after 4th and 8th week (TANITA®, MC-780U, made in Japan). Height will be measured once at baseline with a standard measuring tape, but weight will be measured 3 times, at baseline, after 4th and 8th week (Vitafit®, VT1703U, made in China). IPAQ and 3-day food record will be filled out for participant at baseline and after the 8th week. A total of 10 milliliters of venous blood will be collected from each patient for the assessment of biochemical factors, including CBC/diff (by using Sysmex KX21), FBS, uric acid, creatinine, liver enzymes (AST, ALT, GGT, ALP), lipid profile, albumin (Alb), glycated hemoglobin (HbA1C) (via Auto analyzers) and hs-CRP (by using Colorimetry). The mentioned biochemical will be measured using ELISA kits from isolated serum.

Data Management

The study team will complete specially designed forms at each time point, and these forms will be scanned, reviewed, and entered into a local site database within 48 hours of completion. Data cleansing will be conducted continuously throughout this process. The forms will not contain any patient-identifying information and will be coded using a unique number. Completed forms will be securely stored in a locked folder till the end of the study and will then be destroyed.

Data Analysis

Intention to treat method will be used to analyze the results of this study. In this method, the data of all participants assigned to each study group will be reviewed and analyzed at the end of the study. Quantitative variables will be checked for normality using the Shapiro-Wilk test. Quantitative variables with normal and non-normal distributions will be described using mean ± standard deviation; and median and interquartile range, respectively. In order to compare the quantitative variables between the study groups, the independent t-test (for normally distributed variables) or the Mann-Whitney test (for non-normally distributed variables) will be used. In order to control the confounding variables, the analysis of covariance test will be performed. In order to compare quantitative variables that have been measured more than twice, repeated measurements analysis of variance (ANOVA) will be performed. Qualitative variables will be described using frequency and percentage, and chi-square of Fisher exact tests will be used to compare these variables between the two groups. Statistical analysis will be done using the statistical package for social sciences (SPSS) version 22 software. The level of statistical significance in this study will be 0.05. Patients, researcher, allocator, and evaluator will be blinded.

Discussion

NAFLD is a status where excess fatty acids accumulate in hepatocytes [1]. NAFLD is a chronic disease and it can progress to NASH, fibrosis, cirrhosis and hepatocellular carcinoma if left untreated [2]. The global and national

prevalence of NAFLD (25% and 33.9%, respectively) have raise concern regarding this condition and necessitated necessary preventive and curative actions [3, 4]. Inflammation and insulin resistance play an important role in the development of NAFLD [36].

EA is a polyphenol compound found in fruits like peach, pomegranate, grape, strawberry, raspberry, plum and also green tea [24-28]. The unique properties of EA have attracted attentions. Various studies investigated EA pharmacological proprieties. Animal studies showed that EA was effective in reversing liver steatosis, anti-oxidant status, insulin resistance, inflammation, and hyperuricemia [37-44]. Panchal and et al. showed that EA can ameliorate glucose intolerance, metabolic syndrome, and liver steatosis in overweight/obese rats [37]. Kabelova and etal. Showed that EA ameliorate metabolic parameters in overweight/obese rats with metabolic syndrome (45). EA improved hyperlipidemia and prevented fatty liver in obese, high-fat diet-fed, and transgenic animals with type 2 diabetes through multiple mechanisms. These mechanisms include inhibiting SREBP1a and activating PPARα [44]. Zhang and et al. showed that EA can improve liver steatosis induced by v-akt murine thymoma viral oncogene homolog (AKT)[40]. Al Tamimi et al. showed that EA can ameliorate liver steatosis in streptozotocin-diabetic rats. Also, they showed that EA could decrease cholesterol, TG and free fatty acids in both the liver and serum. On the other hand, EA can reduce the level of IL-6 and TNF-α in the liver [44].

Human clinical studies showed beneficial effects for EA on skin, cognition, blood sugar, lipid profile, inflammation markers, irritable bowel syndrome, and polycystic ovarian syndrome [35, 46-49]. Liu et al. showed that EA can improve cognition and lipid profile (cholesterol, TG, LDL-c and HDL-c) in middle-aged overweight men [50]. Ghadimi et al. showed that 200 mg/d EA administration for 8 weeks improved glycemic status (blood sugar, HbA1C, insulin and insulin resistance), antioxidant status (total antioxidant capacity, superoxide dismutase, glutathione peroxidase, and malondialdehyde activity), and inflammatory factors (IL-6, TNF-α and C-reactive protein) in patient with type 2 diabetes mellitus [48]. Since effectiveness of EA on patient with NAFLD has not been investigated, our study will evaluate the effectiveness of EA on liver steatosis in patients with non-alcoholic fatty liver disease. The strength of this study is its randomized double-blind design and considering both the physical activity and diet in outcome assessment. Lack of complete adherence to the diet in some participants is limitation of this research. However, it should be noted that this is not a limitation but rather one of the limitations of lifestyle modification studies, because according to the previous studies, only 30% of patients participating in lifestyle modification programs for the treatment of non-alcoholic fatty liver were able to at the end of one year, achieve a desired weight loss of more than 5% (51).

Conclusion

We evaluate the effects of EA on clinical and paraclinical outcomes in patient with NAFLD. To achieve this goal, we present the protocol for a clinical study. We anticipate that the administration of 200 mg/d EA for 8 weeks will ameliorate liver steatosis and inflammation in patient with NAFLD. The results of the present study, whether positive or negative, would provide reliable scientific evidence that can influence the current and future policies regarding the administration of EA as a complementary treatment in patients with NAFLD.

Declarations

Ethics Approval and Consent to Participate

Data collection will not begin until local ethical approval is obtained. Before randomization, informed consent will be obtained from all patients or their legal guardians, if applicable. The Research Ethics Committees of the Mashhad University of Medical Sciences approved this study proposal (ref approval no. IR.MUMS.MEDICAL.REC.1401.712). Also, study was registered in Iran's clinical trial website and the IRCT code was received with ID IRCT20180103038199N16.

Availability of Data and Materials

The study dataset will be securely stored locally at the Nutrition Department, School of Medicine, Mashhad University of Medical Sciences, Iran, for long-term data storage and access. Datasets will be accessible for all principal investigators upon written request to the corresponding author.

Conflicts of Interest

The authors declare that they have no competing interests.

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Authors' Contributions
MN, VA and MMA designed study protocol; MMA will conduct the study; MMA wrote the study protocol; statistical plan is designed by MMA and AJE; final protocol was revised by MN, VA, FR, LG and MMA; MN and VA were responsible for the final content. All authors have reviewed and approved the final protocol manuscript.

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