STUDY OR PROJECT TITLE: Effect of alpha-lipoic acid and Silybum marianum supplementation (LUDLEV®) in conjunction with a Mediterranean diet for the improvement of metabolic-associated fatty liver disease.

RESPONSIBLE INVESTIGATORS:

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BACKGROUND

Metabolic-associated fatty liver disease (MAFLD) is currently the most common chronic liver disease worldwide, affecting approximately 25-30% of the population, making it a significant public health problem. [1] It is characterized by the presence of hepatic steatosis in more than 5% of hepatocytes, as demonstrated by biopsy, imaging, or biomarkers, and at least one of the following criteria 1) overweight or obesity, 2) diabetes mellitus, 3) at least two metabolic abnormalities (waist circumference greater than 90 cm in men and 80 cm in women; blood pressure ≥130/85 mmHg or specific pharmacological treatment; triglycerides ≥150 mg/dL or specific pharmacological treatment; high-density lipoprotein cholesterol <40 mg/dL in men and <50 mg/dL in women or specific pharmacological treatment; fasting glucose ≥100 mg/dL; insulin resistance index ≥2. 5; C-reactive protein ≥2 mg/dL). [2]

The increase in the prevalence of MAFLD is favored by sedentary lifestyles and poor dietary habits, such as excessive calorie and saturated fat intake, which coincide with the rise in overweight and obesity. On the other hand, the new definition of MAFLD has moved away from the dichotomy of simple steatosis and steatohepatitis, as it has been demonstrated that fatty liver disease should not be seen as a duality, but as an evolving process in which tissue accumulation of triglycerides can be initially benign with subsequent progression to chronic inflammation and tissue damage (steatohepatitis). The presence of fibrosis is considered a prognostic marker of MAFLD and may be present to varying degrees, with or without inflammation, characterized by the formation of collagen fibers due to the transformation of stellate cells into fibroblast-like cells. [3]

The hepatic steatosis observed in patients with MAFLD is secondary to excessive synthesis of hepatic triglycerides from fatty acids derived from adipose tissue, de novo lipogenesis, and endocytosis of triglyceride-rich lipoproteins. Furthermore, the excessive triglyceride content in the liver tissue leads to secondary VLDL secretion, which is characteristic of the atherogenic dyslipidemia in patients with MAFLD. These patients also have elevated serum triglycerides, elevated LDL cholesterol, and low HDL cholesterol, explained by the activity of the plasma protein CETP, which

transfers cholesterol from HDL or LDL to triglyceride-rich lipoproteins (TRL). [4,5] Remnant lipoproteins from lipid metabolism and systemic transport have the ability to passively deposit in arterial walls, triggering atherosclerosis. For this reason, the dyslipidemia present in fatty liver is associated with the risk of cardiovascular diseases and mortality. [6,7]

In addition to alterations in hepatic triglyceride metabolism, patients with MAFLD experience systemic metabolic dysregulation consistent with components of the metabolic syndrome. The most frequently associated comorbidities, now considered diagnostic criteria, are the presence of diabetes mellitus and obesity.

Currently, there is no approved pharmacological treatment for MAFLD. However, it has been demonstrated that intervention with Mediterranean and hypocaloric dietary styles, with or without exercise regimens, improves intrahepatic fatty acid content and hepatic inflammation, as reflected by a reduction in transaminase levels, when weight loss of 5 to 7% is achieved. Improvement in fibrosis and inflammation can be observed with a 10% weight loss. [8,9]

Milk thistle (Silybum marianum) is a medicinal plant native to North Africa, Southern Europe, and Southern Russia, which contains flavonolignans including silybin, isosilybin, silidianin, and silicristin. It has been used in traditional Chinese medicine for the treatment of liver and biliary diseases. Currently, it has demonstrated antioxidant, anti-inflammatory, and antifibrotic effects in liver diseases, particularly in fatty liver disease. Its therapeutic effects are attributed to the active component of the plant's seeds, known as silymarin. [11,12] Silibin, the main active component of silymarin, inhibits oxidative stress in the endoplasmic reticulum of hepatocytes and the assembly of the NLRP3 inflammasome through the NAD+/SIRT2 pathway in mice with fatty liver. [13]

On the other hand, alpha-lipoic acid is a cofactor of mitochondrial enzymes that has been considered an antioxidant. Its reduced form, dihydrolipoate, reacts with reactive oxygen species, thereby protecting the cell membrane.[14] Administration of alpha-lipoic acid has been shown to have beneficial effects on adipokine levels and

metabolic disorders such as diabetes and obesity, which are commonly present in patients with MAFLD. It has been demonstrated that the administration of 1200 mg of alpha-lipoic acid in patients with fatty liver disease reduces insulin resistance and serum leptin levels while increasing adiponectin concentration. However, it did not influence liver histology and biochemical markers. [15]

Therefore, our objective is to demonstrate that the administration of the combination of alpha-lipoic acid and Silybum marianum (LUDLEV®) can improve steatosis in patients with MAFLD when associated with a Mediterranean-style diet, while ensuring a favorable safety profile.

RESEARCH QUESTION

Can supplementation with alpha-lipoic acid and Silybum marianum (LUDLEV®) in combination with a Mediterranean diet improve steatosis and fibrosis in patients with MAFLD?

HYPOTHESIS

The administration of the combination of alpha-lipoic acid and Silybum marianum (LUDLEV®) in combination with a Mediterranean diet reduces the degree of fibrosis and steatosis in patients with MAFLD as assessed by kPa and CAP measurements after 24 weeks of treatment.

OBJECTIVES

Overall objective:

To evaluate the effects of administering alpha-lipoic acid and Silybum marianum (LUDLEV® 300 mg/46.2 mg) in combination with a Mediterranean diet compared to

placebo plus a Mediterranean diet on fibrosis and steatosis in patients with MAFLD after 24 weeks of treatment.

Specific Objectives:

· To assess the effects of administering alpha-lipoic acid and Silybum marianum (LUDLEV® 300 mg/46.2 mg) in combination with a Mediterranean diet versus placebo plus a Mediterranean diet on fibrosis and steatosis in patients with MAFLD after 12 weeks of treatment.

· To evaluate changes in fibrosis measured by kPa using FibroScan® elastography after 12 and 24 weeks of administering alpha-lipoic acid and Silybum marianum (LUDLEV® 300 mg/46.2 mg) every 12 hours in combination with a Mediterranean diet, compared to placebo plus a Mediterranean diet in patients with MAFLD.

· To assess changes in the controlled attenuation parameter (CAP) measured by FibroScan® elastography after 12 and 24 weeks of administering alpha-lipoic acid and silybum marianum (LUDLEV® 300 mg/46.2 mg) every 12 hours in combination with a Mediterranean diet, compared to placebo plus a Mediterranean diet in patients with MAFLD.

· To evaluate biochemical improvement after 12 and 24 weeks of administering alpha-lipoic acid and Silybum marianum (LUDLEV® 300 mg/46.2 mg) every 12 hours in combination with a Mediterranean diet, compared to placebo plus a Mediterranean diet in patients with MAFLD.

· To assess weight loss and changes in body composition after 12 and 24 weeks of administering alpha-lipoic acid and Silybum marianum (LUDLEV® 300 mg/46.2 mg) every 12 hours in combination with a Mediterranean diet, compared to a Mediterranean diet alone in patients with MAFLD.

METHODOLOGY

Research Design

Study type: Randomized, controlled, double-blind clinical trial.

Population Definition

Patients regularly attending the outpatient clinic of the Gastroenterology Department of the Instituto de Investigaciones Médico-Biológicas de la Universidad Veracruzana, diagnosed with MAFLD.

Inclusion criteria:

· Subjects treated at the Instituto de Investigaciones Médico-Biológicas de la Universidad Veracruzana, diagnosed with fatty liver.

· Willingness to voluntarily participate in the study and signing of informed consent.

· Age over 18 years, both sexes.

· Patients with evidence of hepatic steatosis and overweight or obesity.

· Patients with evidence of hepatic steatosis, diabetes mellitus, and overweight or obesity.

· Patients with evidence of steatosis plus metabolic risk abnormalities (waist circumference >102 cm in men, 88 cm in women, blood pressure >130/85 mmHg or on specific treatment, triglycerides >150 mg/dL or on specific treatment, HDL cholesterol <40 mg/dL in men, <50 mg/dL in women or on specific treatment, HOMA-IR >2. 5, CRP >2 mg/dL, or prediabetes defined by fasting plasma glucose 100-125 mg/dL, 2-hour postload glucose 140-199 mg/dL, or HbA1c between 5.7% and 6.4%).

· Agree not to start a diet program during the study.

· Agree not to change their current exercise plan during the study.

Exclusion criteria:

- Patients who meet diagnostic criteria for MAFLD with normal weight.
- Patients with terminal illness, cancer, or end-stage renal disease on renal replacement therapy.
- Pregnant women.
- Patients who are unable to understand or follow the study protocol.
- Sensitivity to the components of the formula.

Elimination criteria:

- Patients who do not comply with the follow-up time and drug administration.
- Patients who do not comply with proper drug intake.
- Patients with intolerance or severe adverse effects to the components of the drug.
- Patients who are unable to undergo FibroScan® examination due to technical limitations.

Sample size calculation

It is calculated based on the previous study by Martinez Rodriguez et al.(Clinical and Experimental PharmacologyClin Exp Pharmacol 2014, 5:1) where forty patients with NAFLD were randomized into two groups to receive metformin 1500 mg q.a.d. or metformin 1500 mg q.a.d. + (15 mcg)-methionine (3 mg)-alpha lipoic acid (200 mg) group for 24 weeks, and showed a 70% versus 15% decrease in ultrasound-graded steatosis ($p<0.001$) in patients receiving alpha lipoic acid. Taking into account this difference in proportions, considering a power of 80%, alpha error of 20% and confidence interval of 95%, 20 pairs (40 patients) are required to be randomized. If we consider a loss of 25% (n=10), a total sample of 50 patients would be required.

Interventions

Patients included in the study will be randomly distributed in a 1:1 ratio to receive the two treatment regimens.

1. **Initial assessment and follow-up**

Initial medical assessment including:

1. Complete medical history
2. Hepatic ultrasound showing evidence of hepatic steatosis. The degree of hepatic steatosis will be determined according to Hamaguchi score (≥3 points) classifying it as follows: absent (0 points), mild (1 point), moderate (2 points) and severe (3 points). This scoring system has a maximum sum of 6 points and considers hepatorenal echo contrast, hepatic brightness, deep attenuation and vascular visualization with a specificity of 100% and sensitivity of 91.7%. [16]
3. The risk of fibrosis will be determined according to FIB-4, NAFLD score or APRI. FIB-4 is considered high risk of fibrosis score ≥3.25, indeterminate 1.45-3.25 and absent ≤1.45; in NAFLD score high risk with ≥0.675, indeterminate -1.455 to 0.675 and absent ≤-1.455; in APRI high risk of significant fibrosis with score ≥1.5, indeterminate 0.5-1.5 and absent≤0.5.[17,18,19]
4. Transitional liver elastography with FibroScan® will be performed at baseline, 12 and 24 weeks follow-up to estimate hepatic fatty infiltration according to the controlled attenuation parameter (CAP) and hepatic stiffness in kilopascals (kpa). Considering fibrosis stages the following cut-off points F0 ≤6.4kpa, F1 6.5 to 6.9 kpa, F2 7 to 8.6kpa, F3 8.7 to 10kpa, F3-F4 10.1 to 15 kpa and F4 ≥15kpa. For hepatic fatty infiltration is considered by CAP S0 ≤247, S1 between 248 to 267, S2 268 to 279 and S3 ≥280. 10 representative valid measurements with success rate (IQR) 30% are considered. All measurements will be taken by the same operator.[21,22]
5. At baseline,12 and 24 weeks follow-up the following laboratory studies will be performed: blood biometry,blood chemistry, liver function tests, lipid profile and C-reactive protein, serum insulin, HOMA calculation.
6. The study of viral hepatitis type B and C will be performed at the beginning of the study.

2. Nutritional assessment and follow-up

The nutritional assessment will consist of:

1. Nutritional medical history: anthropometric measurements, basic electrical impedance and nutritional diagnosis.
2. A diet plan will be established with a Mediterranean style diet according to nutritional diagnosis and sex (caloric plans of 1400, 1500, 1600 or 1700 kcal/day). The composition of the diet is designed to receive 20% protein, 50% carbohydrates, 15% monounsaturated fats, 10% polyunsaturated fats, 5% saturated fats, less than 250 mg/day cholesterol and 20-30 g/day fiber.
3. Subsequent nutritional follow-up appointments: will be carried out in person on a monthly basis for 24 weeks.
4. Anthropometric measurements and basic electrical impedance at 12 and 24 weeks of follow up.
5. Test of adherence to the dietary plan at 12 and 24 weeks of follow-up.

3. Randomization by groups

1. Patients will be randomly distributed by systematic sampling into 2 groups (A and B).

- Group A will receive placebo in combination with a personalized low-calorie Mediterranean diet adjusted for age and sex (between 1400 to 1700 kcal/day) for 24 weeks.
- Group B will receive alpha-lipoic acid ethyl esters and silybum marianum (LUDLEV® 300 mg/46.2 mg) every 12 hours in association with a personalized low-calorie Mediterranean diet adjusted for age and sex (between 1400 to 1700 kcal/day) for 24 weeks.

4. Follow-up and surveillance

1. Physical activity: It will be evaluated by means of the international physical activity questionnaire (IPAQ) that categorizes the levels of physical activity in: low (category 1), moderate (category 2) and high (category 3) according to the intensity, frequency and duration of the training. The recommendation will be made not to modify the usual training plan.
2. Nutritional plan: The monitoring will be carried out in a personalized way through a monthly face-to-face nutritional consultation.
3. Pharmacological treatment: The adherence to the pharmacological treatment will be monitored through a monthly telephone interview. Telephone assistance will be available to resolve doubts and report adverse effects.

5. Analysis of the results

The analysis of the results, elaboration of figures and tables will be carried out with the statistical program IBS SPSS version 22.0. Nominal and ordinal variables will be described with frequencies and percentages; continuous and discrete variables with measures of central tendency and dispersion according to their distribution. The normality of data distribution will be evaluated with the Kolmogorov-Smirnov test, and nonparametric statistics will be used to evaluate intergroup differences between nominal variables. Non-parametric statistics will be used for ordinal variables. A p-value of less than 0.05 will be considered statistically significant.

ETHICAL ASPECTS

During its development, this medical research will comply with international standards in accordance with the ethical principles for medical research on human

beings established in the Declaration of Helsinki of the World Medical Association (64th General Assembly, Fortaleza, Brazil October 2013) and with the national legislation in force indicated in the General Health Law: Title Five; Research for Health; Sole Chapter, to the Regulation of the General Health Law on Research: Title Two; Of the Ethical Aspects of Research on Human Beings; Chapter I; Articles 16 and 23.

This is a low risk research and will not be carried out on vulnerable population groups according to the General Health Law Regulations on Research.

It will be controlled by clinical record in total privacy respecting the guidelines established in the Mexican Official Standard NOM-012-SSA3-2012 that establishes the criteria for the execution of research projects for health in human beings and safeguarding the privacy of patients in compliance with the Federal Law for the Protection of Personal Data in Possession of Individuals; Chapter II and the Federal Law of Transparency and Access to Public Governmental Information; Chapters 20 to 22.

Measures will be taken to protect the confidentiality of such data, omitting information that could reveal the identity of individuals, limiting access to the data only to the research team and using a numerical code to identify patients without using names.

SCHEDULE OF ACTIVITIES 2022-2023

ACTIVITIES	APRIL MAY	JUNE JULY	AGO SEP	OCT NOV	DIC ENE	FEB MARCH	APRIL MAY	JUN JUL	AGO SEP
Elaboration and approval of the research project Elaboration of placebo	X	 X							
Patient recruitment - Medical and nutritional evaluation. - Initial laboratory studies - Nutritional counseling to establish a Mediterranean style dietary plan. - Initial FibroScan® study			X	X	X				
Medical and nutritional follow-up and monitoring (12 weeks) - Monitoring of compliance with the nutritional plan and treatment. - Laboratory studies - FibroScan® study					X	X	X		

Medical and nutritional follow-up and monitoring (24 weeks) - Monitoring of compliance with the nutritional plan and treatment. - Laboratory studies - FibroScan® study						X	X	X	
Data processing								X	
Statistical analysis of results								X	
Reporting the results Manuscript writing								X	
Dissemination									X

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