Autonomous University of the State of Mexico

Faculty of Medicine

Clinical protocol:

"Effect of vitamin C, vitamin D and Zinc supplementation on the immune and inflammatory process in type 2 diabetic subjects in Mexico".

Researchers:

Roxana Valdés Ramos, DSc. E-mail: rvaldesr@uaemex.mx
Beatriz E. Martínez Carrillo, PhD. E-mail: martinez_elina9@hotmail.com

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1. Background:

Type 2 Diabetes Mellitus

According to the WHO, there are more than 346 million individuals with diabetes, of which 90% are type 2. Global estimations for the year 2030 predict an epidemic increase that will reach 366 million. According to the National Nutrition and Health Survey of 2006 (ENSANUT2005), there are 6.4 million type 2 diabetic subjects in Mexico.

Family history of diabetes increases two or four-fold the risk of developing the disease. Glucose intolerance or diabetes are present in 15 to 25 percent of first-degree relatives of type 2 diabetic subjects. If one parent is affected, the risk is 38%, increasing up to 60% when both parents are ill. Even though genetic factors are very important, it is clear that diabetes is a multifactorial condition and very heterogeneous, that needs to be addressed at various levels.

During more than 15 years, evidence supporting the hypothesis that chronic inflammation caused by obesity is a risk factor for the development of type 2 diabetes has been generated by researchers, however all the mechanisms involved in the diseases have not yet been understood. There is an increase in IL-6 and TNFα production related to larger fat mass, particularly at the central level, showing a pro-inflammatory profile in these patients.

On the other hand, acute phase proteins and some cytokines interact with a wide range of metabolic pathways including regulation of insulin, functions of adipocytes as well as lipase- lipoprotein, which are key to the development of insulin resistance. The presence of elevated concentrations of acute phase proteins as well as pro-inflammatory cytokines in obese individuals has helped demonstrate that a chronic low-grade level of inflammation leads to insulin resistance, metabolic syndrome and type 2 diabetes mellitus.

Vitamin C

Ascorbic acid participates as a co-factor in multiple reactions, particularly acting as a potent antioxidant, in collagen, neuropeptide and carnitine synthesis, increasing iron absorption, inhibiting histamine release and stimulating the immune system. The main cause of increased requirements of vitamin C in type 2 diabetes mellitus is the high levels of oxidative stress caused by hyperglycemia.

Plasma vitamin C concentrations have been inversely correlated to glycosylated hemoglobin and fasting and postprandial blood glucose and oxidative stress, but not on lipid profiles. Diabetes has also been associated to periodontal disease and vitamin C supplementation together with dental maneuvers has been shown to improve chronic periodontitis in newly diagnosed type 2 diabetic subjects. Vitamin C has also been shown to reduce anxiety levels but not stress and depression scores in diabetes. Three-month supplementation of vitamins C and E decreased hypertension, blood glucose while increasing superoxide dismutase and glutathione levels.

Vitamin D

There is recent evidence in humans and animal models, suggesting that vitamin D may play an important role in the risk of developing type 2 diabetes mellitus. Vitamin D receptors are present in pancreatic β cells and in cells of the immune system. Additional to its well known role as the main regulator of calcium absorption, vitamin D intervenes in the beta endopeptidase calcium dependent cells. Some cohort studies in the United States and one in Finland have reported an association of vitamin D and risk of type 2 diabetes mellitus. Other studies have found an association between serum vitamin D, insulin resistance and cell β dysfunction.

On the other hand, it has been demonstrated that vitamin D constitutes a predictor of death by cardiovascular disease (CVD) in patients with type 2 diabetes mellitus.

Zinc

Zinc has been widely studied in relation to diabetes mellitus, as it acts as an insulin mimetic and has a role in the regulation of oxidative stress, thus its supplementation may benefit patients with type 2 diabetes mellitus.

It has been shown that zinc supplementation may reduce glucose and HbA1c concentrations in diabetic subjects, contributing to an adequate metabolic control of the disease. High levels of serum zinc have been associated to lower risk of CVD in diabetic patients. Zinc supplementation has been suggested as an additional treatment for metabolic control in type 2 diabetic patients as it promotes insulin signalling. Inflammatory cytokines in type 2 diabetes mellitus are associated to zinc metabolism by zinc transporter and metallothionein gene expression.

Gut microbiota

The adult intestinal microbiota is composed of hundreds to thousands of species, dominated by the Bacteroidetes and Firmicutes phyla. This ecosystem is different and unique, since it includes many species that do not exist in another part of nature, which indicates powerful selective mechanisms that have generated a coevolution of the host with its intestinal microbial symbionts (commensal and mutualists). The adult intestinal microbiota is also partially stable, composed of approximately 40 bacterial species (representing 75% of the intestinal microbiota in terms of abundance).

In recent years, much attention has been given to the microbial community within the human body and its association to the immune system as well as to the development of chronic diseases such as obesity and type 2 diabetes mellitus. There are many factors involved in the health of this microbiota, such as genetics, environment, use of medications and particularly diet. Some types of microorganisms in the microbiota may promote inflammation increasing the risk for chronic diseases. Increased microbial diversity has been associated to decreased inflammation. Dysbiosis of gut microbiota has been associated to insulin resistance and glucose homeostasis as well as low-grade inflammation. It has been identified that the effect on insulin metabolism is through the regulation of insulin signalling molecules such as GLP-1 and PYY, which reduce insulin resistance and improvement of β-cell function.

There is not much information on the effect of zinc or vitamin C supplementation on gut microbiota. However, Vitamin D may promote a reduction in opportunistic pathogens and an increase in bacterial diversity in the gut. Vitamin D and its receptor (VDR) help in controlling dysbiosis, maintaining gut tolerance and protecting from chronic gastrointestinal diseases, through regulating microbiota composition and function in the intestine.

Oxidative stress

There is evidence that oxidative stress, defined as a persistent imbalance between the production of highly reactive molecular species (chiefly oxygen and nitrogen) and antioxidant defenses, leads to tissue damage. Oxidative stress results from increased content of reactive oxygen species (ROS) for example: superoxide and the hydroxyl radical, and uncharged species such as hydrogen peroxide. There are data indicating that ROS formation is a direct consequence of hyperglycemia; more recent studies have suggested that increased free fatty acid levels may also result in ROS formation. Because of their ability to directly oxidize and damage DNA, protein, and lipid, ROS are believed to play a key direct role in the pathogenesis of late diabetic complications.

Previous experimental and clinical studies report that oxidative stress plays a major role in the pathogenesis and development of complications of diabetes mellitus. However, the exact mechanism by which oxidative stress could contribute to and accelerate the development of complications in diabetic mellitus is only partly known and remains to be clarified. Hyperglycemia induces free radical production; on the other hand, it impairs the endogenous antioxidant defense system in patients with diabetes. Endogenous antioxidant defense mechanisms include both enzymatic and non-enzymatic pathways. Their functions in human cells are to counterbalance toxic reactive oxygen species (ROS). Common antioxidants include the vitamins A, C, and E, glutathione (GSH), and the enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GRx).

In diabetes, oxidative stress is caused by increased formation of plasma-free radicals and a reduction in antioxidant defenses. Hyperinsulinemia and hyperglycemia may enhance the production of free radicals and induce oxidative stress. There is considerable evidence that oxidative stress plays a key role in insulin resistance, impaired insulin secretion, and many of the complications of diabetes such as micro-/macro-vascular damage. SOD and GPx are antioxidant enzymes that protect the body against active oxygen-free radicals. Some experimental studies showed that Vitamin D may have antioxidant properties by modifying some of the antioxidant enzymes.

2. Problem

Chronic diseases have become one of the most important public health problems in the world, due to the high costs of treatment and control of complications. Changes in human behaviour and lifestyles in the last century have caused an increase in type 2 diabetes mellitus.

Chronic elevation of blood glucose, even in absence of symptoms, causes damage in several tissues, particularly microcirculation in the retina, kidneys and peripheral

nerves, where the diabetic complications are more evident. This has led to an increase in the incidence of blindness, amputations and renal failure in many countries, developed and developing. Individuals with diabetes have a two to four-fold higher risk than the general population of developing cardiovascular disease; between 50 and 80% of patients with diabetes die of heart disease or its complications, which also favour the development of disabilities, decrease in life expectancy and the use of a large amount of the health services' budget.

In Mexico, the situation of the health service system is complex, as there is a co-existence of chronic and infectious diseases. Type 2 Diabetes Mellitus was prevalent in 8 to 9% of the Mexican population and it is estimated to increase to 12.3% by the year 2025. Since 1940, diabetes has been in the list of the first 20 main causes of mortality, but since 2000, it is the first cause of death in women and the second in men (after ischemia). In 2003, diabetes caused 12.6% of all deaths in the Mexican population, with a mean age of death of 66 years. However, the National Nutrition Survey of 2012 (ENSANUT 2012) indicated that 9.2% of Mexican adults have been diagnosed with diabetes. It is also the main cause of medical outpatient attention and hospitalization.

Until now, researchers consider that the aetiology of type 2 diabetes mellitus is unknown. There are several hypotheses that try to explain the origin of the disease, such as that it is a disorder of the anterior hypothalamus and the endocrine pancreas caused by progressive ischemia or that there is an abnormal innervation of the islets. Recently, the study of the innate immune system has thrown some light, as there is increasing evidence of an inflammatory acute phase response induced by cytokines, closely linked with the generation of insulin resistance and diabetes. Other studies have associated these pathologies with the presence of inflammatory and immune biomarkers such as TNF-α, IL-1, IL-6, C Reactive Protein (PCr), Monocyte Chemotactic Protein-1 (MCP-1), syalic acid, leptin, adiponectin, resistin and visfatin.

Next to inflammation, patients with diabetes face greater risk of certain infections, including influenza, pneumonia, and foot infections. These infections are associated with increased morbidity and mortality.

Dysbiosis of gut microbiota has also been associated to insulin resistance and low-grade chronic inflammation in the development of type 2 diabetes mellitus.

There is evidence suggesting that supplementation with vitamin C, vitamin D or zinc may help patients with type 2 diabetes mellitus achieve a better metabolic control, thus aiding in the prevention of complications at various levels.

Hypothesis:

1. Vitamin C, vitamin D and zinc supplementation decreases pro inflammatory cytokines (TNFα, IFN-γ, IL-1β) and improves anti-inflammatory (IL4, IL-6, IL10, TGF-β) in patients with type 2 diabetes mellitus.
2. Type 2 diabetes mellitus patients supplemented with vitamin C, vitamin D and zinc will have a better metabolic control.
3. Microbiota of patients with Type 2 diabetes mellitus will have a better profile after supplementation with vitamin C, vitamin D and zinc.
4. Vitamin C, vitamin D and zinc supplementation decreases oxidative stress levels (MDA and protein carbonyl) and improves antioxidant systems

(antioxidant capacity, enzymatic activity: catalase, superoxide dismutase and glutathione peroxidase) in patients with type 2 diabetes mellitus.

3. Objectives

General objective:
To evaluate the effect of vitamin C, vitamin D and zinc supplementation on glucose metabolism, inflammatory and immune system biomarkers, oxidative stress and gut microbiota in patients with type 2 diabetes mellitus in the State of Mexico.

Specific objectives:

1. To evaluate the effect of vitamin C, vitamin D and zinc supplementation on metabolic control (plasma glucose, HOMA, Hb1Ac and lipid profile).
2. To analyse the effect of vitamin C, vitamin D and zinc supplementation on inflammatory and immune biomarkers.
3. To analyse the effect of vitamin C, vitamin D and zinc supplementation on oxidative stress and antioxidant profile.
4. To evaluate the effect of vitamin C, vitamin D and zinc supplementation on gut microbiota.

4. Methods

Study design
Clinical trial randomized, prospective, placebo-controlled, double blinded, comparing two groups of patients with type 2 diabetes mellitus in the State of Mexico.

According to the calculation of the sample size, we will include 120 adults with type 2 diabetes mellitus selected from the outpatient preventive medicine offices of health centres in the State of Mexico who will divided in two groups: supplement and placebo (60 per group). After having been invited to participate and obtaining the informed consent, study subjects will be evaluated for dietary information, as well as biochemical biomarkers of metabolic control, anthropometric, immune and inflammatory markers, gut microbiota and oxidative stress, before beginning the trial, and after 12 and 24 weeks of supplementation. They will have a monthly follow-up visit for evaluation of adherence and adverse effects, as well as delivery of the supplement.

Study groups

Subjects will be randomly allocated to a supplementation with 1000 mg vitamin C, 400 IU vitamin D and 10 mg of zinc or placebo group, during 24 weeks. Subjects and researchers will be blinded to the supplement or placebo in order to guarantee double-blinding.

Sample size calculations:

Based on a previous study done by this same research group (*Nutrients* **2017**, *9*, 573; doi:10.3390/nu9060573), we calculated the sample size with the data on the effect of a

6-month *n*-3 fatty acid supplementation trial on glucose concentrations of group of subjects with type 2 diabetes mellitus,

The calculations were done with the following formula:

$$N = \frac{(2 \times s^2)\,(Z\alpha + Z\beta)^2}{d^2}$$

Where: $Z_{\alpha/2} = 1.96$, $Z_{\beta} = 0.84$, s = 68.9 mg/dL and d = 40mg/dL

Thus:

$$N = \frac{(2 \times 4747.21)\,(1.96 + 0.84)^2}{40^2} = 46 \approx 50 \text{ subjects}$$

Accounting for a 20% loss at follow-up, we added 10 subjects, resulting in 60 subjects per group. This sample size guarantees that we should be able to observe significant differences in all the other indicators we will measure.

The following criteria will be used for the selection of the study sample:

Inclusion criteria

- Between 25 and 55 years of age, as this is the age in which type 2 diabetes mellitus is more prevalent and there is less probability of encountering multiple diseases in the same subjects
- Both sexes
- Outpatients
- BIM ≥ 25

Exclusion criteria

- Without any other chronic disease (cancer, cardiovascular diseases, arthritis and Alzheimer's).
- Severe renal insufficiency.
- Nephrolithiasis or history of nephrolithiasis.
- Hyperoxaluria.
- Hemochromatosis.
- Hypercalcaemia.
- Hypervitaminosis D.
- Using insulin.
- Be taking drugs such as desferrioxamine, iron, cyclosporine, indinavir (protease inhibitors), warfarin, thiazide diuretic, orlistat, ion exchange resins (e.g cholestyramine, laxatives (e.g. mineral oil, senna), vitamin d analogues (e.g. ergocalciferol, calcitriol, and topical calcipotriene), tetracycline antibiotics, quinolone antibiotics, penicillamine, biphosphonates, levothyroxine, eltrombopag.
- Patients with hypersensitivity to any of the active substance(s) or to any of the excipients.
- Hypersensitivity to the by-products including honey, conifers, poplars, Peru balsam, and salicylate.

- Intake of probiotics or supplemental vitamin or mineral (vitamin D, C, zinc or calcium) for 4 weeks before the beginning of the study.
- Smoking and alcohol consumption (> 40 gr/ day for men and 25 gr/ day for women.
- Pregnant or lactating.
- Whose parents or grandparents are/were immigrant or of native origin.

Measurements.

Subjects will be selected from the outpatient preventive medicine offices of health centres in the State of Mexico. After having been invited to participate and obtaining their informed consent, study subjects will be evaluated for dietary information, as well as biochemical, anthropometric, immune and inflammatory markers, as well as intestinal microbiota before beginning the trial, at 12 and 24 weeks of supplementation.

A qualified physician will do a complete clinical history. Subjects will be requested to fill-out the Diabetes 39 quality of life questionnaire (validated in Mexico), at baseline and at the end of the study. Questions on frequency of respiratory, urinary and gastrointestinal infections as well as on perception with respect to the supplement will be included.

A standardized nutritionist will evaluate Dietary History, a food-frequency questionnaire (FFQ), as well as three 24-hour food registry of non-consecutive days including one from the weekend. The FFQ will be analysed in DIAL software to evaluate dietary intake of vitamin C, vitamin D and zinc. Anthropometric evaluations will be done by a standardized nutritionist; weight will be measured with a 1631 TANITA™ portable electronic scale, stature with a 1013522 SECA™ portable stadiometer, for the calculation of BMI.

Faecal samples will be obtained for evaluation of intestinal microbiota. We will proceed to isolate and characterize the bacteria obtained from the faecal samples with two enriched culture media for the purification of the most representative and abundant strains. The isolated strains will be classified in morphological groups according to their macroscopic characteristics of size, form, surface, consistency and color. A selection of 40% of the representative samples from each morphologic group will be typified by the RFLP method. We will obtain biomass and extract DNA, for amplification of the 16S rRNA gene, with an enzymatic genotypification and strain grouping. The strains will be grouped to compare their diversity; the results of the groups will be compared to appreciate the differences between the type and number of strains obtained.

At each sampling date, a venous blood sample of 8.5 mL will be obtained after a 12 hour fast for the measurement of vitamin C (HPLC); zinc (Atomic Absorption Spectrometry); vitamin D and Vitamin D binding protein (ELISA); plasma glucose; triacylglycerides; total cholesterol; HDL and LDL-cholesterol and Hb1Ac (Automated Selectra equipment with Randox™ reactants); pro inflammatory cytokines (TNFα, IFN-γ, IL-1β) and anti-inflammatory cytokines (IL4, IL-6, IL10, TGF-β), apolipoproteins A and B, C-reactive protein, V-CAM, I-CAM, complement proteins C-3 and C-4, insulin, adiponectin, resisitin and leptin will be analysed with multiplex technology (Milliplex Luminex™).Additionally we will measure biomarkers of oxidative stress and

antioxidant systems such as Malondialdehyde (QuantiChrom™ TBARS Assay Kit), protein carbonyls (calorimetric assay), antioxidant capacity (QuantiChrom™ Antioxidant Assay Kit), Catalase activity (EnzyChrom™ Catalase Assay Kit), Superoxide Dismutase activity (EnzyChrom™ Superoxido Dismutase Assay Kit) and Glutathione Peroxidase (metaphosphoric acid SIGMA ALDRICH and EnzyChrom™ GSH/GSSG Assay Kit).

HOMA-IR and HOMA-B

$HOMA_{IR}$ will be calculated as follows: $HOMA_{IR}$ = (insulin x glucose)/405

HOMA-B will be calculated as follows: HOMA-B = 20 × insulin (μUI/mL) / (glucose [mmol/L] – 3,5) (160).

Follow-up

Additionally to their monthly visits to the clinic, the researchers will maintain close communication via telephone or e-mail with all participants, to promote adherence, and to identify any possible adverse effect of the supplements. Adherence will be evaluated by the Morisky-Green and Haynes-Sackett tests.

The investigators will collect, document, report and follow-up all events that meet the definition of an adverse event (AE) or serious adverse event (SAE) to the Health Authority (COFEPRIS) or the Ethics Committee in accordance with local legal obligations. AEs/SAEs will be collected from the first day the subject consumes the supplement up to three months after end of supplementation.

Adverse effects will also be registered during the monthly follow-up visits. If an adverse effect that puts the subject at risk is identified, such as an allergy, the supplementation will be suspended and reported to the authorities (COFEPRIS), as well as the producers of the supplement.

Bioethics

This project will be conducted according to the Declaration of Helsinky and all its amendments, including the one in 2013. We will also abide by the General Health Law of the Mexican Ministry of Health with respect to research in human beings.

The protocol will be reviewed and approved by the Research and Ethics Committees of the Faculty of Medicine of the Universidad Autónoma del Estado de México.

Participation in the study will be totally free and voluntary, subject to the participants' authorization by the signature of a letter of informed consent.

The procedures in the present study are considered with a risk higher than minimum, due to the need to draw blood by venipuncture.

All information will be maintained confidential.

Data Collection and Statistical Analysis

A specific database will be designed in SPSS 19.0®. Data will be described with means and standard deviations or frequencies and percentages depending on the type of data. We will undertake a normality analysis, after which differences between groups will be analysed by parametric or non-parametric statistical tests. A multiple regression analysis will be run in order to establish interactions between variables.

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