EZEKIEL PROJECT

Study of micronized olive leaves (mol) and phenolic compounds: osteoblastic stimulation, osteocalcin synthesis and bone formation. Prevention of postmenopausal Osteoporosis and Spaceflight osteoporosis.

Ezekiel 47,12
"And by the river upon the bank there of on this side and on that side, shall grow every fruitful tree for food, whose leaf shall not fall, neither shall its fruit be lacking; it shall bring forth mature fruit in its months, because their waters come forth out of the sanctuary; and its fruit shall be for food, and its leaf for medicine"

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ABSTRACT
The project aims to identify and analyze the unexplored therapeutic potential offered by the administration of a pool of phenolic compounds derived from the oleic leaves of the olive tree (*Olea europaea* L.) in micronized form: Micronized Olive Leaves (MOL), on diseases of bone metabolism and replacement, particularly on the prevention of osteoporosis.
To develop this design idea, we will gradually articulate some steps: the general dietary nutrition to the Mediterranean diet and its basic nutritional properties, the role of phenolic compounds on bone tissue, and in which the functional contribution of phenols and the effects on bone metabolism utilize the role of osteoblastic stimulation with the production of osteocalcin (OC) as a potential therapeutic agent in the formation of the bone matrix.

STUDY PHASES
a. Finding the pedo-environmental and agronomic contexts and the varieties of *Olea europaea* more suitable for the study of the secondary metabolites of the leaves. Prof. Giovanna Abbate, Dr. Mauro Iberite

b. Study of secondary metabolites contained in Micronized Olive Leaves (MOL). Dr. Cinzia Benincasa

c. Study of the Effects of Micronized Olive Leaves (MOL) on osteocalcin (OC) circulating levels (age, sex, weight and dietary habits). Dr. Roberto Cuccurello

d. Ultrastructural morphological study by electronic microscopy for scanning and transmission of tissue from mice. Prof.Giuseppe Familiari, Dr. Michela Relucenti

e. Synthesis of some of the compounds identified by proteomic analysis to test their activities individually and in combinations. Synthesis of phenolic derivatives as suggested by ultrastructural morphological study, that will be tested to study the effects on osteocalcine. Dr. Paolo Bovicelli, Dr. Alessandra Ricelli

f. Morphometric and biomechanical study of the bone structure. Verification of MOL administration on the increase in the amount of osteocalcin (OC) circulating levels. Prof. Franco Marinozzi, Dr Rossella Bedini.

Introduction

1. Phytonutrients: Mediterranean diet, olive and phenols

Constant adherence to the nutritional characteristics and the nutrition requirements of the Mediterranean Diet (MD) is significantly associated with a lower incidence of Alzheimer's disease and neurodegenerative disorders as well as on the onset of cardiovascular disease.
A Mediterranean Diet is made up of a rich intake of fatty acids (mainly derived from olive oil), complex carbohydrates in the form of cereals and legumes, a high fiber content, in particular

fruit and vegetables, but also a limited use of animal proteins. In this context, extra virgin olive oil (EVO) has long been the subject of special attention due to its various potential benefits. As the main component of Md, EVO contains variable amounts of triacylglycerols and lower amounts of free fatty acids, glycerol, pigments, sterols and non-fully-characterized resinous components. The nutraceutical properties of the products obtained from the fruit, especially fruits and leaves, have recently been recognized as important components in various therapeutic actions thanks to their high phenolic input (1).

Regular EVO consumption has been shown to improve some cardiovascular risk factors such as lipid profile, blood pressure, endothelial dysfunction, oxidative stress and antithrombotic profile.

As an example, in the context of neurodegenerative disorders, there is evidence that the Consumption of EVOO in animal model of amyotrophic lateral sclerosis is able to improve the motor performance and the status of muscle trophism by expressing myogenic factors and therefore reducing muscular damage.

Olive (*Olea europaea*) is the native plant of the Mediterranean region whose main bioactive components include phenolic constituents.

As phenol derivatives, phenolic compounds are substances consisting of a benzene ring which may present as substituents one or more hydroxyl groups and functional groups such as glucose to which most of the phenols are conjugated. This generally makes phenolic compounds, essentially hydrophilic molecules more than lipophilic, although it is possible to divide the lipophilic and hydrophilic phenols. The phenolic compounds of EVO are schematically classified as: 1) phenolic acids; 2) phenolic alcohols; 3) secoiridoids; 4) lignans; 5) flavonoids and 6) tocopherols.

Among the best characterized and main phenolic compounds, we find: hydroxytyrosol (HT; 3,4-dihydroxyphenylethanol; 3,4-DHPEA) and oleuropein (Ole). While HT is a phenolic alcohol, Ole is a secoiridoid. Phenolic compounds are found throughout the olive plant, yet their nature and concentration vary greatly between the various varieties and constituent parts.

In Olea europaea, Ole, dimethyl-Ole, ligstroside and oleoside represent the predominant phenolic oleosides. In particular, Ole represents the phenolic compound prevalent in the olive and can reach high concentrations per Kg of dehydrated raw material (2). It has been shown that the variation in the concentration (from about 440 to about 180 g / Kg) depends on the cultivar of the olive plant as well as the maturation phase (3). Several different functional properties are attributed to Ole, including antioxidants (1), anti-inflammatory (4), anti-atherogenic (5), anti-cancerous (6), anti-microbial (7) and anti-viral (8). In addition, Ole seems to possess cardiovascular properties (9) as well as anti-ischemic, ipolipidemic (10) and neuroprotective activity as suggested by both in vitro and epidemiological evidence where the positive impact of phenolic extracts on the incidence of aging-related disorders like dementia. In one of these studies (11), it has been shown that Ole may reduce or even prevent the β amyloid protein aggregate involved in the pathogenesis of Alzheimer's disease.

2. Phenolic Compounds and Bone Remodeling

Despite the different functional properties identified and linked to the synergic action of phenolic compounds, and in particular Ole, these compounds remain poorly investigated, especially in some clinically relevant clinical physiopathologic and epidemiological impacts on public health and the economy of the national healthcare system, such as osteoporotic pathology.

Reduction of bone mass represents an invariable feature of human biology affecting man and woman and constitutes an important determinant of osteoporosis and the consequent risk of

bone fractures and disability. Bone tissue reduction is associated with a lower osteoblastic activity during bone remodeling than osteoclastic remission attributable to a combination of several factors, including genetic, metabolic, hormonal, nutritional factors and related to physical activity. The incidence of osteoporosis is lower in the Mediterranean basin than in the rest of Europe (12), and this supports the idea that the major source of phenolic compounds constituted by regular EVO consumption should be further investigated in its nutraceutical mechanisms. In spite of the important therapeutic potential of the phenolic compounds of the olive plant, studies of their preventative effects on bone metabolic diseases, such as osteoporosis, are still scarce.

In this regard, however, it is encouraging the data that in animal models of osteoporosis senile consumption of EVO, as well as Ole (15 mg / kg), stops bone remodeling and prevents bone loss (13-15). A recent study has shown that Ole can enhance osteoblastogenesis by also stimulating the mineralization of the extracellular matrix produced by osteoblasts, limiting bone reabsorption and thus preventing bone loss (16). The high phenolic content and the prominent antioxidant and anti-inflammatory properties of these compounds (17) are generally called into question as mechanisms underlying the potentially preventative action of bone mass loss.

3. Phenolic compounds and bone metabolism: an osteocalcin-dependent pathway

Regarding the protective and potentially regenerative role exercised by the phenolic compounds derived from the olives on bone tissue, the longitudinal impact of daily EVOO associated with a regular Md (Md + EVOO) or Md associated with consumption of dried fruit (nuts) (Md + nuts) on the circulating levels of osteocalcin (OC), as a marker of regeneration of bone tissue (18).

Well, the combination of Md and EVOO but not that between Md and nuts has been shown to induce a significant increase in plasma OC concentration even after two years of dietetic supplementation (18). However, due to the different purposes of this study, essentially oriented to the assessment of cardiovascular risk, no specific effects were observed on the health of bone tissue resulting from Md + EVOO supplementation as effects on bone mineral density or in reducing the risk of fractures.

Just recently (19), 64 osteopenic patients participating in a clinical trial in blind patients underwent the voluntary daily intake of an olive phenolic extract (250 mg / day; Bonolive®) associated with calcium supplements (Ca, 1000 mg / day). At the end of the 12 months of expected treatment, an increase in OC was observed as a pro-osteoblastic marker in the Bonolive® + Ca group with an unchanged bone mineral density (BMD) profile in the face of its deterioration in the control group (19).

These preliminary indications disclose the possibility of systematically investigating OC-induced therapeutic potential on bone reshipment. Currently osteoporotic therapy is largely based on osteoclastic bone resorption-inhibiting activity. However, this requires clarifying which role and functional placement finds the OC within bone metabolism. OC is a hormone derived from osteoblasts and therefore intimately linked to the formation of new bone tissue, is secreted in the extracellular matrix of the bone as well as in the general circulation. OC or GLA or BGP (Bone Gamma-carboxyglutamate Protein) is one of the most abundant non-collagen proteins of bone tissue. This small peptide (49 amino acids in humans, 46 in the mouse) is produced by osteoblasts during bone formation and therefore its serum concentration is a biochemical marker of bone genesis (bone turnover). OC is post-transcriptionally modified on three specific gamma- carboxyglutamic acid residues (positions 17, 21 and 24) with vitamin K as a co-factor that allows the carboxylation of the three glutamic residues.

The acidic environment generated during the bone resorption process promotes the decarboxylation of the OC (GlaOC) trapped in the bone matrix by generating its non-carboxyl

form (GluOC) which alone performs hormonal function. GluOC has been shown to play a key role in glycemic and energy metabolism (20) and brain development (21). The skeleton and bone tissue require a constant supply of energy to remodel and maintain the necessary structural strength to prevent, but also to repair, any damage (fractures).
The constant need for energy input and use for remodeling as well as ability
of the bone tissue to dynamically adapt form and size to the needs of mechanical work (such as obesity or, in contrast, physical exercise) demonstrate the need for constant bi-directional communication between bone tissue and energy metabolism.

Spaceflight osteoporosis

Osteoporosis from space flights is a little-investigated aspect as the use of bone-stimulating compounds. Use of MOL may be effective as osteoblastic stimulation and may be crucial in preventing this type of osteoporosis.
It may be interesting to work with international research centers using the protocol described for our research (25-26-27)

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