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Relation Between Probiotic Milk Administration and Some Bone Turnover Markers

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Abstract: The evaluation of several biochemical markers can reflect the overall skeletal rate of bone turnover. Scarce information about the effects of probiotic foods on the calcium metabolism and bone turnover markers is available. A fermented milk containing probiotic lactobacilli, as a natural strategy to prevent or slow the bone loss process in perimenospausal women, was assayed. A randomized double-blind study with apparently healthy adult women was undertaken to assess whether an improvement of markers of bone turnover levels can be achieved after oral administration of lactobacilli. The results obtained from urinary Deoxypyridinoline levels on day 0 and day 35 indicated significant differences ($p<0.05$) between perimenopausal women receiving probiotic and those receiving placebo. The urinary type I collagen cross-linked N-telopeptide, did not showed significant differences ($p<0.05$) between day 0 and day 35 of women included in Test group. In each woman of Control group the relation calcium/creatinine did not show significant differences ($p>0.05$) between day 0 and day 35. The results obtained from two bone formation markers, serum osteocalcin and bone-specific alkaline phosphatase did not show significant differences ($p>0.05$). The results obtained in the present work could infer that the fermented milk consumption decreased the calcium loss in adult women.

Key words: Probiotic lactobacilli, Bone turnover, Perimenopausal women

Introduction

Bone is a specialized connective tissue composed of cells and an extracellular matrix. (Termine, 1993). Its metabolism is characterized by two opposed processes: formation and resorption. The formation depends on osteoblasts and the bone resorption depends on osteoclasts. The combination of these two processes is defined as bone remodeling (Baron, 1993). Formations and resorption processes appear to be very well coupled in normal conditions, to the point that the equilibrium between both will determine the gain, loss, or balance of bone total mass.

Bone mass in the middle and later years is dependent on achieving an ideal peak bone mass and on one's success in slowing age-related bone loss. The majority of bone mass is developed during the adolescent and young adulthood years, with nearly 90% of skeletal mass accumulated by age 18. Current research has demonstrated that young women's intake of calcium is significantly below the recommended dietary intake (Kass-Wolff, 2004).

There is a genetic component to peak bone mass, but external or environmental conditions, such as adequate diet, determine whether an individual reaches his or her potential genetic. Risk factors of osteopenia are non-modifiable (premature menopause, small frame, positive family history, etc.), or modifiable (calcium intake, high alcohol, cigarette use, inactive lifestyle, etc.) (Piper *et al.*, 1992). The increase in bone turnover rate with an imbalance between formation and resorption generally began at pre or peri-menopausal period and persist in postmenopausal women.

The understanding of the physiopathology of the postmenopausal bone loss has improved significantly during the last decade and has placed the role of bone turnover in focus as and independent risk factor for osteoporosis. Increased bone turnover results in bone loss a perhaps deteriorated bone structure. The bone turnover is reflected by concentration in serum and urine of either enzymatic activities of the bone cells involved in the turnover processes or by products, which are released during bone formation or degradation. The so called "biochemical markers of bone turnover" have been rapidly improved in specificity and sensitivity within the last 5 years (Christiansen *et al.*, 1998).

Earlier studies have shown that the biochemical markers of bone turnover can be used to estimate both short-and long-term bone loss, either alone or in combination with bone densitometry measurements. Bone formation markers, total and serum Osteocalcin and total and bone specific alkaline phosphatase, are direct or indirect

products of active osteoblast expressed during different phases of osteoblast development and reflecting different aspects of their function and formation. Most biochemical markers of bone resorption are degradation products of bone collagen and urinary excretion of hidroxiproline: total and peptide bound and free-pyridinium cross-links (Ravn *et al.*, 1996).

Because the osteoporosis is a mayor health problem and it is being increasingly important in our aging society. To combat osteoporosis are urgently needed, but the way of doing so is uncertain. The current goals in the prevention of osteoporosis are to improve the peak bone mass attained subgroups of the population in risk (as in adolescents pre-, peri- and post-menopausal women) of disease; and alternatively, the intervention aimed at the all population, in others to maintain bone mass. This includes promotes behavior modification as altering lifestyles with respect to general nutrition and calcium intake, maximizing physical activity, and reducing or eliminating risk factors that are detrimental to bone.

The current trend is to use *probiotic foods* as prevention and/or therapy in human and animals diseases. The meaning of *probiotic* was the object of a long debate, all authors agree on the direct or indirect benefits of these products on the health of humans, animals and plants. Naidu *et al.* (1999) have proposed a definition for probiotic foods: living microorganisms which, when consumed in a certain amount, have effects on health that go beyond basic inherent nutrition. Probiotic foods can be administered to human or animals in order to prevent diseases, to strengthen the barrier function of the gut microflora and/ or for a non-specific enhancement of the immune system (Sögaard and Suhr-Jessen, 1990).

There have been few carefully designed studies of the effects of dairy food (milk, yogurt, cheese) intake on bone health. The aim of the present work was to study, in perimenopausal woman, the effects of probiotic milk on bone metabolism, as a natural strategy to prevent or slow the bone loss process.

Materials and Methods

Patients: Forty elderly (40-50 years olds) ambulatory female voluntaries were selected by professionals of the Medical Center of Humberto 1°, Santa Fe - Argentine. The women were recruited by questionnaires and medical screening examinations. In order to obtain samples of healthy perimenopausal women without secondary causes of osteoporosis, women with diseases and medical treatment known to affect skeleton were excluded.

Their general conditions were stable, regular menses, and they were free of known malignant diseases. Other inclusion criteria were: no previous or present history of diseases affecting calcium or bone metabolism, absence of diabetes, normal thyroid function, no medication (including oral contraceptives), no previous bone fractures (Ravn, *et al.*, 1996).

Microorganisms: *Lactobacillus casei* CRL 431 and *Lactobacillus acidophilus* CRL 730 strains were isolated from feces of healthy children and characterized in CERELA. The strains were cultured at 37°C in MRS broth (Oxoid Ltd., U.S.A.) (de Man *et al.*, 1960). All strains were kept at -20 °C in MRS broth with 20 % glycerol.

Methods

A randomized double-blind study with apparently healthy adults was undertaken to assess whether an increase of calcium levels in blood can be achieved after oral administration of lactobacilli. Dietary calcium intake was assessed by means of a food frequency questionnaire that inquired about weekly consumption of dairy products.

Women were randomized into two treatment groups to receive daily supplements of: (a) placebo milk, constituted the Control group; and (b) probiotic milk constituted, the Test group.

Food Supplementation: Test-milk was prepared carried by addition of individual concentrated of washed lactobacillus cells to sterile 13% reconstituted milk. The product was used after 4h incubation at 37°C (final concentration of 10^7 lactobacilli/ mL). Equal amounts of both cultures were mixed together and kept at 4°C. This preparation was performed once a week and here-after it is called fermented milk. Unfermented (placebo) and fermented milks preparation was carried out in CERELA. Afterward both products under refrigeration conditions were submitted to Humberto 1° Medical Center. Each patient received daily 240 ml of skim milk added with either 10 ml of placebo (Control group) or fermented milk (Test group) and this food supplementation was administered during 1 week; the following 7 days the patients didn't receive food supplementation. The treatment consisted in 5 weeks with alternated food supplementation.

All participants were put in observation at the same time and their blood and urine samples were taken before (day 0), during (days 7, 14, 21, 28) and the end food supplementation (day 35). The samples were stored at -20° C until processing.

Markers of Bone Turnover: Blood samples were collected between 8-9 a.m. after an overnight fast. For each woman, a fasting 2h morning urine samples and a 24h urine sample were also collected. The following serum

biochemical determinations were performed:
Bone formation markers: Serum intact osteocalcin (OC) (Diagnostic System Laboratories, Texas, USA) using an immuradiometric assay (IRMA). Bone-Alkaline phosphatases (B-ALP) were measured with Alkphase-B which used monoclonal antibodies (Garnero and Delmas, 1993 and Garnero *et al.*, 1994).

Bone resorption markers: Urinary type I collagen cross-linked N-telopeptide (NTX), and urinary Deoxypyrydinoline (Dpd). Urinary NTX was measured with an ELISA test using monoclonal antibody against the N-telopeptide to helix intermolecular cross-linking domain of type I collagen isolated from human urine (Hanson, 1992). The urinary Deoxypyrydinoline was measured with a Pyrilinks-D (Metra Biosystems). This assay is a competitive enzyme immunoassay utilizing a monoclonal anti-Dpd antibody (Delmas, 1995).

Statistical Methods: The changes in biochemical markers of bone turnover with time during treatment with fermented milk and placebo were evaluated by analysis of variance with unpaired Student´s *t* test (Rossman and Chance, 1998)

Results and Discussion

Calcium is the major cation of bone mineral. The rate of calcium removal from the skeleton tends to parallel the calcium deposition rate. The rate of calcium accumulation in the skeleton is the difference between deposition and removal rates and it constitutes the bone calcium mass. The bone calcium mass of the newborn infant is quite small and reaches a maximum in persons 35-45 years old. After that age, bone calcium mass decreases, gradually in men and abruptly in women for about a decade after their menopause (Piper *et al.*, 1992; Bronner and Pansu, 1999). Bone remodeling is a coupled process, and biochemical markers of bone remodeling help establish the rate of bone turnover and are a useful adjunct for predicting future rates of bone loss, as well as the response to specific therapies. These markers usually fall in a normal range and if elevated are more likely to indicate a rapid rate of bone loss, as well as identify those individuals more likely to respond to antiresorptive agents.
Nevertheless it is apparent that when calcium intake is adequate, differences in bioavailability, as from increased solubilization, play no or only a minor role in the amount of calcium that is absorbed or deposited in the skeleton (Deroisy *et al.*, 1997 and Tsugawa *et al.*, 1995). When, however, the dietary calcium content is low and in the form of poorly soluble or poorly digestible sources, e.g., spinach, the decrease in calcium absorption compared to a source like milk becomes nutritionally significant. The high absorbability of calcium in milk has been related to the presence of lactose, phosphopeptides and amino acids, the latter perhaps derived from hydrolysis of casein in the intestinal lumen. It is of interest that some milk substitutes, though otherwise nutritionally equivalent, are compared to milk, a poorer source for calcium and phosphorus (Fournier, 1954; Mykkänen and Wasserman, 1980).
Evidence has been presented indicating bioavailability of calcium, zinc, iron, manganese, copper and phosphorus is increased in yoghurt compared to milk. Those findings are supported by the observation that the growth rate differential between rats fed yoghurt and milk can be reduced by supplementation of milk with minerals especially iron, zinc, manganese and copper (McDonough *et al.*, 1983). It is commonly held that bone loss is largely due to decreased intestinal calcium absorption. Relatively little is know about the effects of probiotic foods on the calcium metabolism and bone turnover markers in humans.
Probiotic strains can be selected by their conditions of normal intestinal habitants of host and their beneficial properties (Havenaar *et al.*, 1992). The probiotic spectrum of activity can be divided into nutritional, physiological, and antimicrobial effects (Naidu *et al.*, 1999 and Rowland *et al.*, 1997).
In our laboratory, we isolated two lactobacilli strains, *Lactobacillus casei* CRL 431 and *Lactobacillus acidophilus* CRL 730, from the faeces of healthy children. We used them to prepare fermented milk for the prevention and therapy of diarrhea (González *et al.*, 1990) and demonstrated their protective effect against different pathogens (Apella *et al.*, s1992 and Nader de Macías *et al.*, 1993). These lactic acid bacteria produced an immunostimulant effect in mice (Perdigón and Alvarez, 1992). Information on the cell wall structure of these bacteria to explain their biotherapeutic behaviors was carried out by Morata de Ambrosini *et al.* (1998; 1999).
Although menopausal status and hormone replacement therapy use dominate women's bone health, diet may influence early menopausal bone loss.
Preliminary assays consisting in oral administration of fermented milk to healthy female volunteers aged between 20-30 years olds demonstrated similar calcium levels in blood in Control and Test groups, however the same study, but performed in healthy female volunteers aged between 40-50 years old indicated higher serum calcium levels in patients included in Test group than those included in Control group (Ortiz Zavalla *et al.*, 1997).
However, in this work we analyzed in perimenospausal women, the effects of probiotic milk on bone metabolism. Two bone resorption markers were studied, the osteocalcin (OC) and bone-Alkaline phosphatase (B-ALP). The OC is a protein exclusively synthesized by osteoblasts, odontoblast and hypertrophic chondrocytes. This protein is considered to be specific marker of osteoblasts function, as its levels correlate with bone formation rates (Seibel,

2000). However, the peptide is rapidly degraded in serum and both intact peptides and OC fragments of varies sizes coexist in circulation. This above mentioned, results in limitations in the clinical applications of this priori specific marker (Delmas *et al.*, 2000). Alkaline phosphatase (ALP) is a ubiquitous enzyme that plays an important role in osteoid formation and mineralization. The total ALP serum pool consists of several dimeric isoforms which originate from various tissues such as liver, bone, intestine, spleen, kidney and placenta. From a clinical perspective, however, detection of the bone specific ALP (Bone ALP) isoenzyme is increasingly preferred because of its higher specificity (Delmas *et al.*, 2000).

The results obtained on serum OC and B-ALP are shown in Fig.s 1 and 2. We did not observed differences in OC levels in Test group at ending probiotics treatment, but only observed significant differences between perimenopausal women receiving probiotic and those receiving placebo when OC levels was measured at 28 and 35 days (Fig. 1). The results of B-ALP levels did not suffer modification with probiotics administration (Fig. 2). Therefore, in each patient of Test group the relation calcium/creatinine was lower on day 35 than day 0, while this relation was not modified in women of Control groups (data not shown). However, these results obtained indicated that oral administration of fermented milk was unable to produce enhance of bone formation.

The results of determination of urinary deoxypyridinoline (Dpd) and urinary type I collagen cross-linked n-telopeptide (NTX) are shown in Fig. 3 and 4. The organic matrix of bone consists almost of 90% type I collagen, which gains structural rigidity from pyridinium cross-linking between the adjacent collagen fibrils. This cross-links, deoxypyridinoline and pyridoline are formed from lysine and hydroxylysine residues during the maduration of collagen matrix. This cross-linking occurs between the helix of one collagen molecule and the non-helical amino-(N) or carboxyterminal (C) telopeptide ends of an adjacent collagen molecule. After bone degradation, cross-links are released into the circulation and excreted in urine as a mixture of free and peptide-links forms. The free forms of the

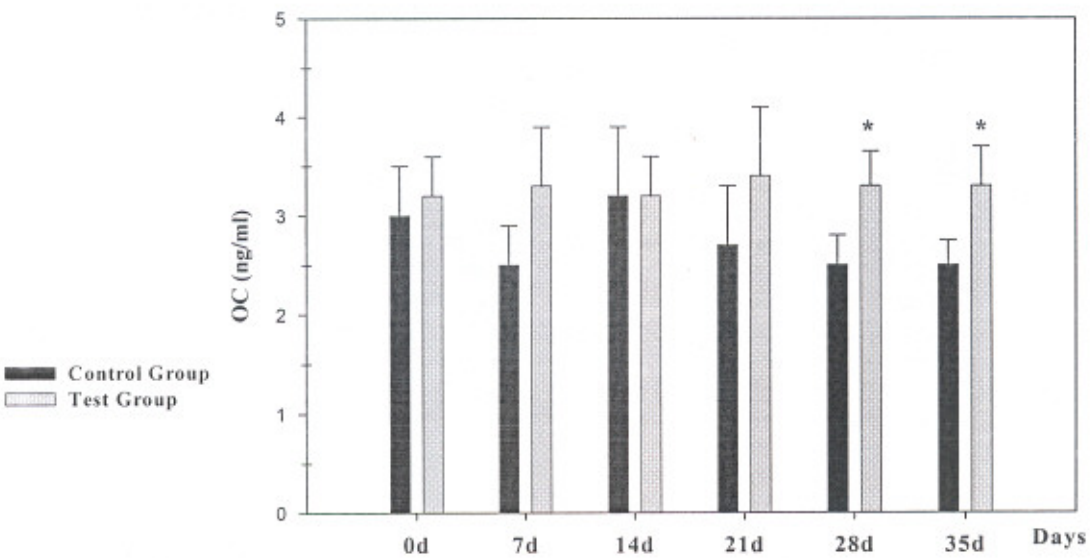

Fig. 1: Effect of fermented milk on bone absortion in perimenopausal women.

The Fig. represents the determination of serum Osteocalcin in Test (fermented milk) and Control (unfermented milk) group during 5 weeks of treatment.
All values was expressed as media ± SD from n = 20 women in each group.
* Significantly differences between Test and Control group ($P<0,05$).
OC: serum osteocalcin

pyridinium cross-links can be measured as free deoxipyridinoline (Dpd). The peptide-linked forms contain N- and C- telopeptides of collagen (Np and Cp respectivelly). Urinary Dpd, Np and Cp have proven to be specific and sensitive bone resorption markers for evaluation of osteoporosis (Sorva *et al.*, 1994).

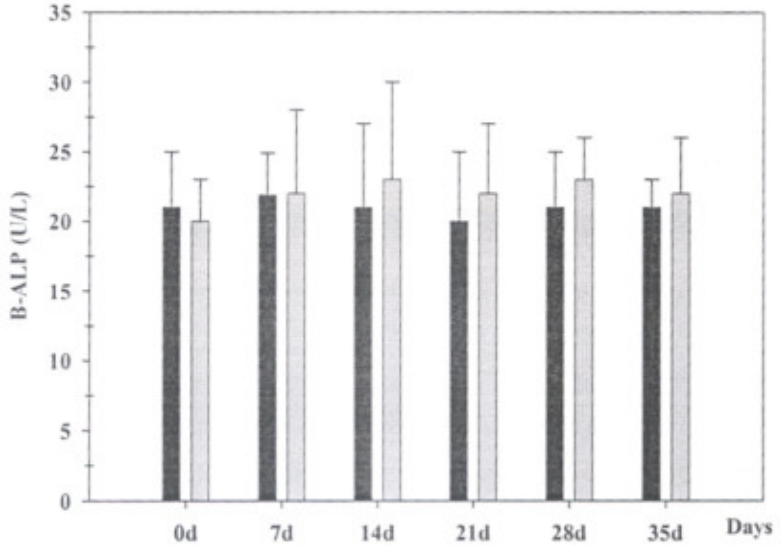

Fig. 2: Effect of fermented milk on bone absortion in perimenospausal women. The Fig. represents the determination of serum bone Alkaline phosphatase in Test (fermented milk) and Control (unfermented milk) group during 5 weeks of treatment.
All values was expressed as media ± SD from n = 20 women in each group.
There was not significant difference between Test and Control group.
B-ALP: serum bone Alkaline phosphatase

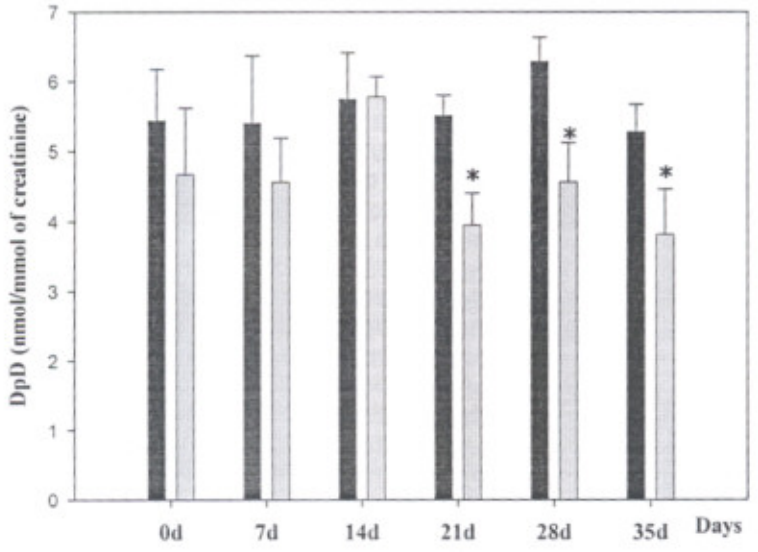

Fig. 3: Effect of fermented milk on bone absortion in perimenospausal women. The Fig. represents the urinary Deoxypyridinoline levels in test (fermented milk) and control (unfermented milk) group during 5weeks of treatment.
All values was expressed as media ± SD from n = 20 women in each group.
* Significantly differences between Test and Control group ($P<0,05$).
DpD: urinary Deoxypyridinoline levels

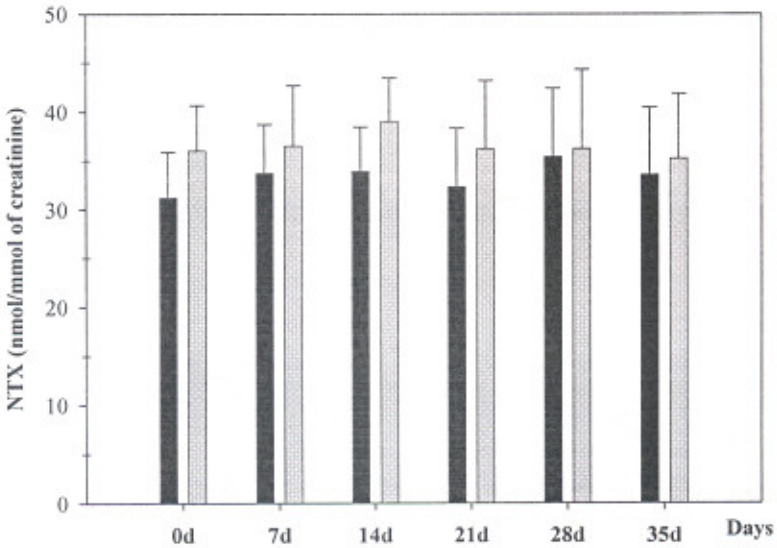

Fig. 4: Effect of fermented milk on bone absortion in perimenospausal women.

The Fig. represents the urinary N-telopeptide levels in test (fermented milk) and control (unfermented milk) group during 5 weeks of treatment.
All values was expressed as media ± SD from n = 20 women in each group.
There was not significant difference between Test and Control group.
NTX: urinary N-telopeptide levels

When we analyzed the levels of Dpd we observed a significantly decrease of this resorption bone marker from 21 days between perimenopausal women receiving probiotics and control group (Fig. 3). The other bone resorption marker measured, NTX, did not sowed significant difference between both studied groups (Fig. 4).
Although it is still debatable whether there are any changes in bone turnover in the perimopausal period; however, once markers have increased at the menopause they remain elevated and generally do not change with age (Delmas *et al.*, 2000). Keeping in mind that mentioned, ratifies the importance of avoiding the increment of the bone markers in the perimenopausal women by a feeding strategy with probiotics.
From the results obtained in the present work it could infer that fermented milk consumption is able to decrease the calcium loss because it prevents the bone resorption/ formation disturbance, because we observed that the probiotics feeding induces a descent of one resorption bone marker.
However, we have not obtained results indicating increase of formation bone during the time of probiotics administration assayed in this work. Possibly, this happens because the calcium absorption stimulated by probiotics feeding is not high so that to increase the bone formation. Although, others authors showed that fermentation of milk with *Lactobacillus helveticus* had a positive acute effects on calcium metabolism (Narva *et al.*, 2004), there is substantial evidence to suggest that all dairy foods are not equivalent vehicles of calcium, perhaps because of their different protein, sodium, potassium, and vitamin A contents. Dairy protein contributes to bone loss, in part because of the generation of fixed acid. As reservoir of labile base as calcium salts, the skeleton may provide neutralization at expense of structure. Dairy food intake is closely linked to protein intake; it is relate to calcium excretion, bone resorption, and bone fracture risk (Weinster and Krumdieck, 2000).
A prebiotic is definite as "a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon". The probiotic facilitate the colonic absorption of minerals, particularly Ca^{2+} and Mg^{2+} (Roberfroid, 2000). However, we suggested a combining probiotics and prebiotics in what has been called a symbiotic could beneficially affect the calcium absorption.
This paper includes preliminary studies about probiotic food and bone turnover, other assays from high number of patients, during prolonged time and testing at beginning and ending with bone densitometry are necessary to establish the effect of probiotic administration on resorption/formation activity in perimenopausal women.

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