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Casein hydrolysates enhance osteoblast proliferation and differentiation in mouse bone marrow culture

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ABSTRACT

Osteoporosis is characterized by low bone mass with micro-architectural deterioration of bone tissue leading to enhanced bone fragility, thus increasing the susceptibility to fracture. Osteoporosis is most common in women after menopause, and is referred to as postmenopausal osteoporosis. Extensive scientific evidence has been provided for the existence of biologically active peptides derived from milk that may have beneficial effects on bone health. In the present study casein was separated from buffalo milk and was hydrolyzed with trypsin to form casein hydrolysates. Angiotensin Converting Enzyme (ACE) inhibitory activities of casein hydrolysates were measured and its effect on osteoblast proliferation and differentiation was studied. Casein hydrolysates at 50 and 250 μg/mL concentration stimulated the proliferation and differentiation of osteoblasts *in vitro* which was confirmed by MTT proliferation assay. Casein hydrolysates also increased calcium mineralization in osteoblast cells in comparison to control. Likewise, the expression of osteocalcin, osteoprotegerin and alkaline phosphatase genes which are known marker of osteoblast differentiation has also been significantly ($P<0.05$) increased in treatment groups as compared with the control. These results showed that, the casein hydrolysates with ACE inhibitory activity are anabolic to bone, an effect that is consequent upon their potent proliferative and differentiated actions in osteoblasts.

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1. Introduction

There are about 300 million people with osteoporosis in India and 80% of them are women. On a global basis, Indians have the highest prevalence of osteopenia (Malhotra & Mithal, 2008). Compared to Caucasians, osteoporotic fractures in the Indian population occur 10–12 years earlier in age (Unni, Garg, & Pawar, 2010). Osteoporotic fractures are more common in Indian men than in the Western country (Eisman, 2004). These facts dictate an urgent need to address issues relevant to the prevention of osteoporosis. To meet the challenge of dealing effectively with this major public health problem the only solution is by preventing bone loss (Unni et al., 2010). There are three major components involved in an effective preventive strategy. Maximizing peak bone mass during childhood, adolescence and early adulthood is the first preventive step. Although much of peak bone mass is determined by genetic influence (60–70%) but there are other factors of importance over which one has control. These include adequate dietary calcium intake, proper nutrition, physical activity and hormone sufficiency. Maintaining the bone mass is the second aspect of prevention. The

Abbreviations: ACE, Angiotensin Converting Enzyme; *alp*, alkaline phosphatase; *ocn*, osteocalcin; *opg*, osteoprotegerin

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maintenance of bone requires adequate calcium intake and exercise as well as avoiding tobacco and alcohol. Certain diseases like hyperthyroidism (Yang, Shen, Zheng, & Zhang, 2012) and medications (steroids, anticonvulsants) tend to erode the repositories of bone (Ensrud et al., 2008; Ledford, Apter, & Brenner, 1998).The third aspect to prevention is counteracting the process of age-related bone loss that occurs after 40–45 years of age. In women, the menopause markedly accelerates bone loss. Measures to ensure that bone loss is minimized during the middle years and beyond include adequate nutrition (vitamin D and calcium) and hormone sufficiency (Kulak & Bilezikian, 1998).

Milk is a good source of several nutrients, such as protein, calcium, phosphate, magnesium, potassium and zinc that are considered important for bone health. In epidemiological studies milk consumption in childhood and during adolescence has been related to higher bone mineral density in adulthood (Kalkwarf, Khoury, & Lanphear, 2003; Murphy, Khaw, May, & Composton, 1994). Intervention studies show that milk and milk products prevented the bone loss in pre-menopausal and post-menopausal women (Chee, 2003). Dairy products have also been shown to increase not only bone mineral density but also bone growth (Cheng et al., 2005; Lanou, Berkow, & Barnard, 2005; Matkovic et al., 2004). In recent years extensive scientific evidence has been provided for the existence of biologically active peptides derived from milk proteins that may have beneficial effects on human health. The beneficial health effects may be classified as antioxidative, antithrombotic, antihypertensive, antimicrobial or immunomodulatory (Haque, Chand, & Kapila, 2009; Korhonen & Pihlanto, 2006). Milk protein derived bioactive peptides are inactive within the sequence of parent protein and can be released by enzymatic proteolysis. Many of the known bioactive peptides have been produced *in vitro* using gastrointestinal enzymes usually pepsin and trypsin and by fermentation with bacteria (Daniela et al., 2010). Angiotensin Converting Enzyme (ACE) inhibitory peptides for example are most commonly produced by trypsin (Lopez-Fandino, Otte, & Camp (2006)). Recently, Kwok et al. (2012) reported that ACE-inhibitors inhibit bone loss in an old man. Narva, Halleen, Vaananen, & Korpela (2004) also previously reported that ACE-inhibitory peptides have a positive impact on bone health. But, it is not clear whether the osteogenic effect of bioactive peptides is mediated by regulating osteoblast proliferation or differentiation. So, there is a need to investigate further the anabolic effect of milk derived bioactive peptides on osteoblast proliferation and to clarify their role in osteoblast differentiation as well. Keeping this in view, the present study has been conducted to find out the effect of casein hydrolysates with ACE inhibitory activity on proliferation and differentiation of osteoblasts *in vitro*.

2. Materials and methods

2.1. Preparation of casein hydrolysates depicting ACE inhibitory peptide

Buffalo (*Bubalus bubalis*) milk was procured from Cattle Yard, National Dairy Research Institute, Karnal, India. Milk was defatted, warmed to 40 °C and casein was precipitated by lowering pH to 4.6 (Behera et al., 2012). The precipitate so formed was separated, washed four times with distilled water and α-, β- and κ-caseins were fractionated by following the protocol of Fox and Guiney (1972). These isoelectric caseins were dissolved separately in tris buffer (Tris–HCl 0.02 M, $CaCl_2$ 10 mM, pH 8.0) at the rate of 5 mg/mL and hydrolyzed with trypsin. In brief, enzyme was added at the ratio (trypsin: casein) of 1:100 and incubated at 37 °C for 10 h. The reaction was stopped by heating at 98 °C for 10 min, cooled and centrifuged at 10,000*g* for 20 min at 5 °C. The supernatant was collected and stored at −20 °C. Hydrolysates having molecular weight less than 3 KDa were fractionated by centrifugal ultrafiltration (Bhattacharjee, Bhattacharjee, & Datta, 2006). Angiotensin Converting Enzyme Inhibitory (ACEI) activity of casein hydrolysates were measured by following the protocol of Cushman and Cheung (1971) and modification of Hernandez-Ledesma, Amigo, Ramos, and Recio (2004) protocol.

2.2. Animals

Female albino mice of Wistar strain (10 weeks old) were obtained from Small Animal House of National Dairy Research Institute (NDRI), Karnal. The experimental protocol was approved by Institute Ethics committee of Animal Experiments (IEAE), National Dairy Research institute, Karnal, India. The mice were housed under conventional conditions maintained at 21 ± 1 °C, with 40–60% humidity, and an inverse artificial light–dark cycle of 12 h (light period from 0800 to 2000 h). Mice were kept in uniform nutritional condition in a control diet containing 45% starch, 25% Bengal gram, 5% sucrose, 10% soybean oil, 1% vitamin mix, 4% mineral mixture, 0.2% choline chloride and 0.35% methionine. Vitamin and mineral mixtures were prepared and blended according to AOAC (1984).

2.3. Osteoblast cell culture

Bone marrow cells from femur of female albino mice of Wistar strain were cultured in sterile 60 mL tissue culture bottle containing Dulbecco's modified eagle (DME) Hams F-12 medium supplemented with 10% fetal calf serum, 10 nM dexamethasone, 50 μg/mL ascorbic acid, 50 μg/mL L-glutamine, 10 mM β-glycerophosphate, 100 IU/mL penicillin and 100 μg/mL streptomycin. The pH of the medium was adjusted to 7.4 with 4.4% sodium bicarbonate solution. Prior to addition of fetal calf serum, media was filtered through Millipore membrane (0.22 μm). Bone marrow cells were cultured at a density of 1×10^6 cell/cm^2 along with different concentrations of casein hydrolysates in a CO_2 incubator (5% CO_2, 37 °C, 80% relative humidity) for 21 days. Half of the medium was carefully removed and replaced with fresh medium on every third day. Thus, only part of the non-adherent cells were removed during each medium change (Sila-asna, Bunyaratvej, Maeda, Kitaguchi, & Bunyaratavej, 2007). After 21 days of culture osteoblasts were stained with alizarin red-S dye to see calcium mineralization in cells. Alizarin is an anthraquinone derivative, used to identify calcium deposition in osteoblasts. Calcium forms a complex with alizarin

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