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Essential amino acids increase the growth and alkaline phosphatase activity in osteoblasts cultured in vitro

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Abstract

An inadequate protein intake seems to be involved in the pathogenesis of osteoporosis. Moreover, protein from animal sources appears to protect against hip fracture, while protein from vegetable sources, which present low levels of essential amino acids, has no effect. In this preliminary work, the growth, the alkaline phosphatase activity and the collagen synthesis were evaluated in osteoblast cultures obtained from calvaria of newborn Sprague–Dawley rats and incubated with lysine, threonine, methionine, triptophan and arginine. Our results have shown that the essential amino acids can modulate the growth and the differentiation of osteoblasts cultured in vitro, confirming the relationship between osteoporotic hip fracture and inadequate protein intake. The compounds have mainly enhanced cell growth and alkaline phosphatase activity, and, to a lower degree, collagen synthesis. In summary, the essential amino acids can stimulate bone formation and could represents useful agents for the prevention and therapy of osteoporosis.

Keywords: Essential amino acids; Osteoblasts; Collagen synthesis; Alkaline phosphatase activity

1. Introduction

Several factors, such as age, hormone status and diet are implicated in the pathogenesis of osteoporosis, a disease characterized by progressive bone loss. Actually, osteoporotic hip fracture represents a severe health problem in terms of both morbidity and financial costs [1].

Malnutrition and undernutrition, due to an inadequate food intake, malabsorption and increased metabolism [2], cause a deficiency in both micronutrients and macronutrients [3].

Several studies [4–6] showed that a relationship exists between bone health and nutrition in all age categories, especially in the elderly population. Low protein intake induces a decrease in bone density of femoral neck [7], while protein supplementation improves the medical outcome of hip fracture patients [8]. Moreover, protein intake can influence the calcium balance in young adults [9]. Recently Munger et al. [10] reported that protein from animal sources appears to protect against hip fracture, while protein from vegetable sources has no effect. This observation could be explained with differences in availability between animal and vegetable sources, probably due to associated dietary components that can affect the absorption and the metabolism of amino acids. An alternative interpretation could be found in differences in protein quality: vegetable proteins present low levels of essential amino acids. In particular, the amino acid lysine plays a role in the cross-linking of both collagen and osteopontin [11]. So an inadequate intake of this amino acid induces a decrease in collagen synthesis and an increase in risk of fractures [12].

In order to better clarify the role of some essential amino acids in bone health, the growth, the alkaline phosphatase activity, and the collagen synthesis were evaluated in osteoblast cultures obtained from calvaria of newborn Sprague–Dawley rats and incubated with lysine, threonine, methionine, triptophan and arginine.

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2. Experimental

2.1. Cell culture

Osteoblasts were isolated from calvaria of newborn Sprague–Dawley rats by enzymatic digestion. Calvariae were dissected aseptically, stripped of loosely adherent soft connective tissue, washed three times in phosphate-buffered saline (PBS) containing the following antibiotics, 100 IU/ml penicillin G, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B (Sigma, St. Louis, MO). Then the minced calvariae were sequentially digested six times in a solution containing 0.1% collagenase B (Roche Diagnostics, Milan, Italy), 0.05% trypsin (Sigma) and 4 mM EDTA disodium salt (Sigma) in PBS at 37 °C for 30 min. The cells obtained were plated and incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 in MEM (Life Technologies, Milan, Italy)/F12 (Sigma) 1:1 containing 2% Ultroser (Life Technologies), 1 mM sodium pyruvate (Life Technologies), 2 mM glutamine (Sigma), 5 μM non-essential amino acids (Life Technologies), 50 μM ascorbic acid (Sigma) and 1% antibiotic solution.

For all experiments osteoblasts, in two to three passages, were incubated for 48 and 72 h with L-arginine (Arg), L-lysine (Lys), L-methionine (Met), L-threonine (Thr) and L-triptophan (Trp) (Sigma).

2.2. MTT assay

Osteoblasts were seeded in 96-well tissue culture microtiter plates (5×10^3 cells/well). After 24 h, the medium was removed, replace with fresh medium without sodium pyruvate and non-essential amino acids and supplemented with Arg, Lys, Met, Thr and Trp at dosages equivalent to 0.1-, 1-, 10-, 100-fold their plasmatic concentrations (Table 1).

Following a 48- and 72-h stimulation period, MTT assay was performed as described by Mosmann [14]. Briefly, 10 μl of the MTT solution (5 mg/ml) (Sigma) was added per 100 μl of culture medium and the plates were incubated for 4 h; 100 μl of acid propan-2-ol (0.04 M HCl in propan-2-ol) was added. After a few minutes shaking, the plates were read on a microplate reader (Mod. EL13 Bio-Tek Instruments Inc. Winooski, VT, USA) at a wavelength of 570 nm. The optical density (OD) values were converted into a growth percentage; the OD value corresponding to the non-treated cultures was taken as 100%. The linearity of absorbance of MTT over a range of 1×10^3–4×10^4 cells was established by determining the linear coefficient. Each experiment was repeated six times.

Table 1
Human plasmatic concentration of essential amino acids [13]

Amino acid	Plasma concentration (μmol/l)
Arg	75
Lys	190
Met	30
Thr	150
Trp	44

2.3. Alkaline phosphatase activity

Osteoblasts were seeded in 24-well tissue culture plates (2×10^5 cells/well). After 24 h from seeding, the medium was replaced with fresh medium without sodium pyruvate and non-essential amino acids, and supplemented with Arg, Lys, Met, Thr and Trp at doses equivalent to 0.1-, 1-, 10-, 100-fold their plasmatic concentrations. After 48 and 72 h, the medium was removed and the cells were lysated with a solution containing 1% Triton X-100 (Sigma); at the same time cellular alkaline phosphatase activity and cell protein were determined.

A total of 0.1 ml of cell lysates was placed in tubes and mixed to a solution containing 0.25 ml of 2-amino-2-methyl-1-propanol, 1.5 M, pH 10.3, and 0.25 ml of *p*-nitrophenyl phosphate disodium (4 mg/ml) (Sigma). The tubes were placed in a 37 °C water bath for 2 h and then the absorbances were read on a microplate reader (EL13) at a wavelength of 405 nm. The OD values were converted into Sigma units (a Sigma unit of phosphatase activity is defined as that amount of enzyme that liberates 1 μmol of *p*-nitrophenol per hour) by a calibration curve previously prepared according to Sigma protocol.

The activity was adjusted to the cell protein as determined with Bicinchoninic acid protein assay (Pierce, Rockford, IL) and expressed as Sigma units/mg of total proteins. The values were converted into a percentage: the activity value corresponding to the non-treated cultures was taken as 100%. Each experiment was repeated three times.

2.4. Collagenase-digestible protein synthesis

Osteoblasts were seeded in 24-well plates at a density of 2×10^5 cells/well. After 24 h from seeding, the medium was replaced with fresh medium without sodium pyruvate and non-essential amino acids, and supplemented with Arg, Lys, Met, Thr and Trp at doses equivalent to 0.1-, 1-, 10-, 100-fold their plasmatic concentrations. Following a 45-h stimulation period, 4 μCi/ml of L-[5-H^3]proline (Amersham Pharmacia Biotech, UK; specific activity: 25 Ci/mmol) was added for another 3-h incubation. Then, the media were transferred to glass tubes and cell layers were extracted with 0.2 N NaOH (0.5 ml/well) and pooled with the media. Labelled proteins were precipitated

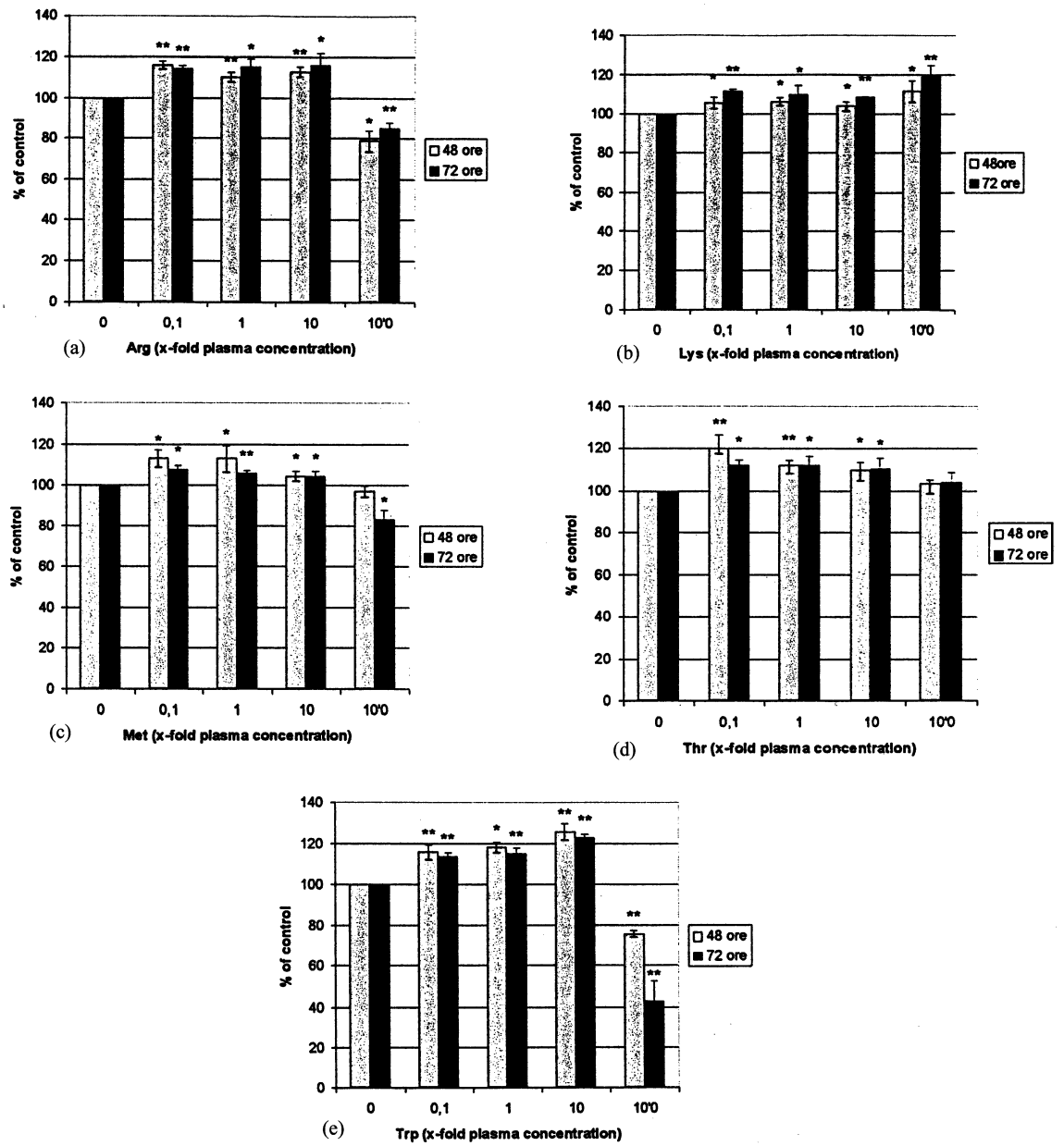

Fig. 1. Effects of Arg (a), Lys (b), Met (c), Thr (d), and Trp (e) on proliferation of osteoblasts. * $P < 0.05$ vs control/** $P < 0.001$ vs control; Student's t-test.

with 0.2 ml of iced 50% trichloroacetic acid (TCA) (Sigma) and 5% tannic acid (Sigma). After centrifugation, the pellets were rinsed out three times with iced 10% TCA and 0.5% tannic acid and then twice with ice-cold acetone. The pellets were resuspended in 0.5 ml of 0.05 N NaOH, neutralized with 0.375 M HCl and buffered with a 20 mmol/l Tris buffer, pH 7.4 (containing 100 mmol/l of $CaCl_2$ and 2 mmol/l of N-ethylmaleimide). The solution was divide in two aliquots: one tube from each pair received 25 U collagenase (Roche, 2.82 U/mg) and the remaining received an equal volume of buffer. After 3 h of incubation at 37 °C, the reaction was stopped by addition of iced 50% TCA and 5% tannic acid. After centrifugation, the pellets were collected and resuspended with 2 N NaOH. Labelled protein was counted with a Minaxi β Tricarb 4000 Series spectrometer (Packard Bioscience B.V., Groningen, The Netherlands). Collagenase-digestible protein synthesis was calculated as the difference of insoluble [H^3]proline, with and without collagenase. The amount of collagen synthesized, expressed as a percentage of total protein synthesis, was calculated using a factor of 5.4 in order to correct for the relative abundance of proline in collagen compared to the non-collagen protein [15]. Final results were expressed in percentages, taking as 100% the non-treated cultures. Each experiment was repeated three times.

3. Results

3.1. MTT assay

Arg (Fig. 1a), Met (Fig. 1c), Thr (Fig. 1d), and Trp (Fig. 1e) induced significant increases ($P < 0.05$ and $P < 0.001$) in cell growth at doses equivalent to 0.1-, 1-, and 10-fold plasma concentration. All concentrations of Lys (Fig. 1b) enhanced cell proliferation, with a maximal stimulation with Lys of 100-fold.

Arg and Trp appeared more effective in increasing cell growth than the other amino acids. Arg, Met, and Trp 100-fold induced significant decreases ($P < 0.05$ and $P < 0.001$) in cell proliferation compared to that observed in non-treated cultures.

Moreover, no relevant increases in growth were observed ranging from 48 to 72 h of incubation, and no dose–response relation was visible.

3.2. Alkaline phosphatase activity

Alkaline phosphatase activity was significantly enhanced ($P < 0.00$l) with all amino acids at doses equivalent to 0.1-, 1- and 10-fold plasma concentration (Fig. 2). These increases were more visible for Lys (Fig. 2b), Met (Fig. 2c), Thr (Fig. 2d), and Tpr (Fig. 2e) after a 48-h incubation period compared to those observed after 72 h of incubation. Nevertheless, no differences were revealed between the two treatment periods with Arg (Fig. 2a).

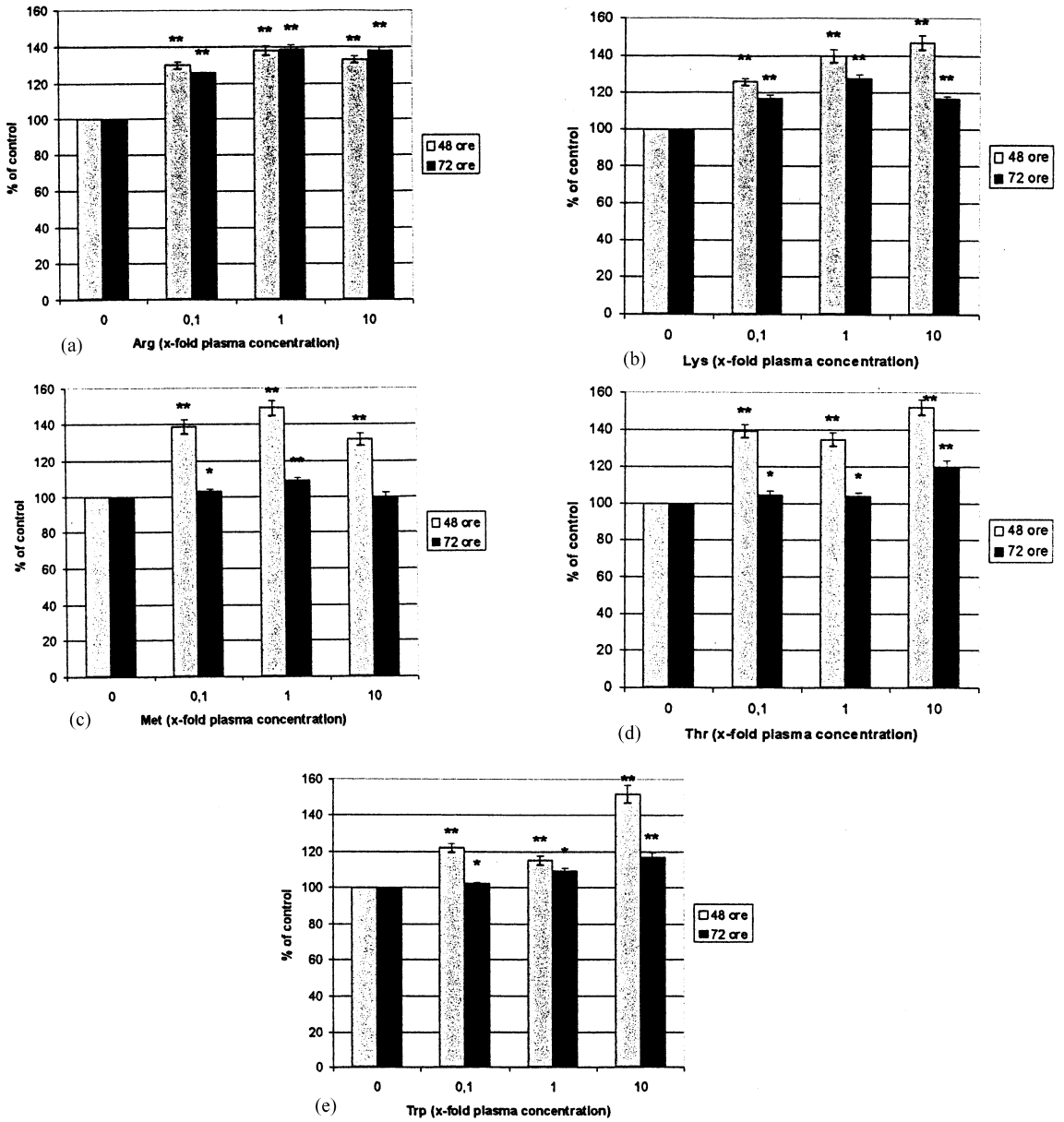

Fig. 2. Effects of Arg (a), Lys (b), Met (c), Thr (d), and Trp (e) on alkaline phoshatase activity of osteoblasts. * $P < 0.05$ vs control/** $P < 0.001$ vs control; Student's t-test.

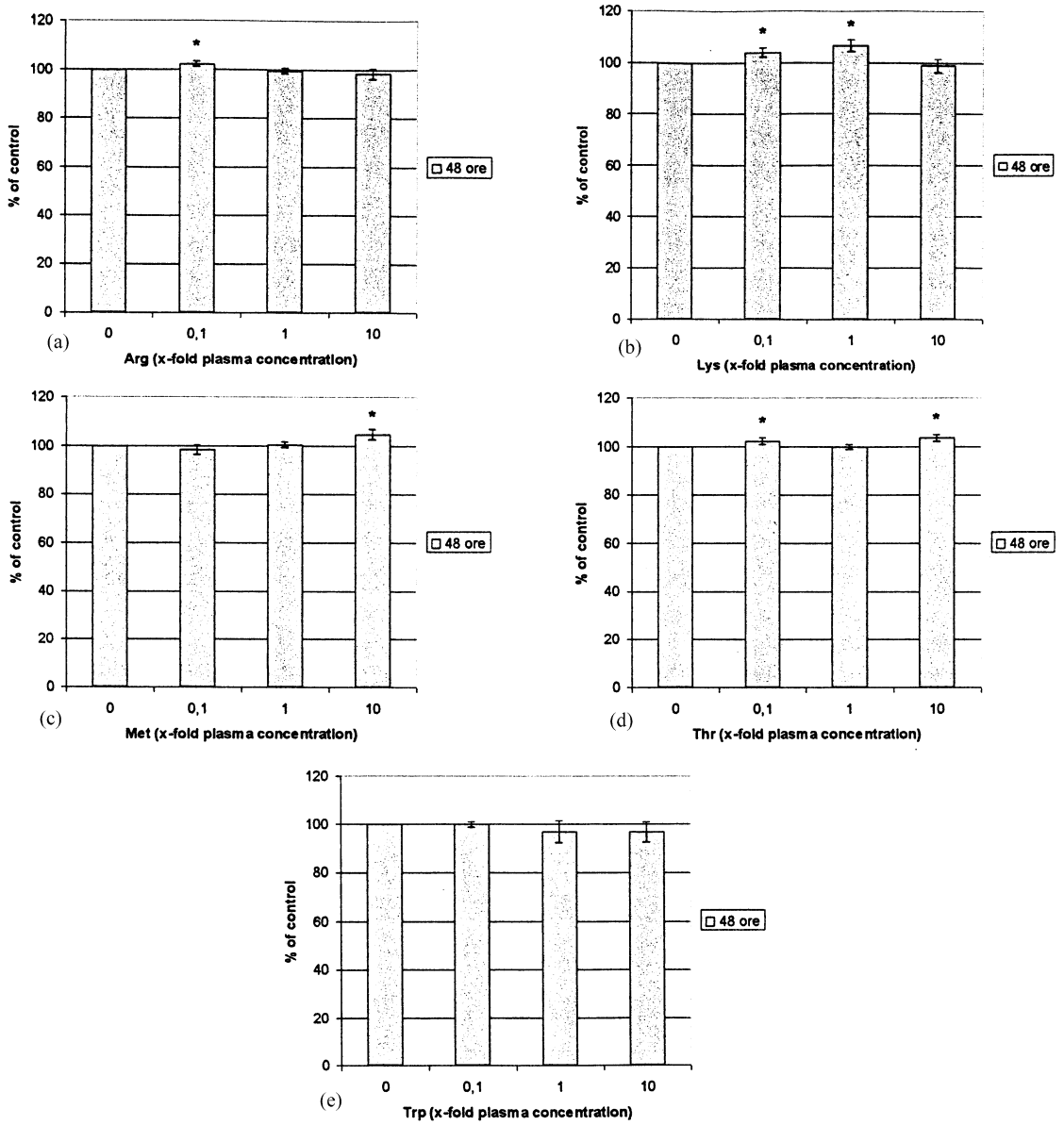

Fig. 3. Effects of Arg (a), Lys (b), Met (c), Thr (d), and Trp (e) on collagen synthesis of osteoblasts. $P < 0.05$ vs control; Student's t-test.

Maximal stimulations were obtained with Met 1-fold and Lys, Thr, and Trp 10-fold. No evident dose–response relations were visible, except for Lys.

3.3. Collagen synthesis

After 48 h of incubation, Arg (Fig. 3a), Lys (Fig. 3b), Met (Fig. 3c), and Thr (Fig. 3d) induced a significant increase ($P < 0.05$) in collagenase-digestible proteins. Nevertheless, slight changes were observed: the percentage of collagen synthesis was maximal (106.7 ± 2.3) with Lys 1-fold. Trp had no effect. After a 72-h incubation period similar results were obtained (data not shown).

4. Discussion

Our results have shown that the essential amino acids can modulate the growth and the differentiation of osteoblasts cultured in vitro, confirming the relationship between osteoporotic hip fracture and inadequate protein intake [4].

The compounds have mainly enhanced cell growth and alkaline phosphatase activity and, to a lower degree, collagen synthesis.

All amino acids have presented mitogenic activity at doses equivalent to 0.1-, 1-, and 10-fold plasma concentration. Similar results were obtained by Chevalley et al. [15] for Arg and Lys. Nevertheless, the decrease in

cell growth observed with Arg, Met, and Trp 100-fold are unclear. Thr and Lys 100-fold, whose plasma value are higher than those of the other amino acids, have induced, respectively, none and maximal increase in cell proliferation. So, the effects of Arg, Met and Trp couldn't be due to an osmotic imbalance.

Moreover, no amino acid enhanced cell growth in a dose-dependent manner. Chevalley et al. [15] observed a dose–response relation only between the Arg 0.1- and 10-fold plasma concentrations.

Our results suggest that the essential amino acids can play a role in bone matrix formation: actually, they have stimulated alkaline phosphatase activity and collagen synthesis.

Alkaline phosphatase is involved in bone mineralization, allowing calcium deposition into the bone matrix. So, the stimulation of its activity represents an useful index to evaluate the influence of amino acids on bone formation. All the compounds have increased this parameter at doses equivalent to 0.1-, 1-, and 10-fold plasma concentration, according to a previous study [15]. Moreover, maximal stimulations have been observed after a 48-h incubation period, except for Arg. The extension of Arg activity could be due to the Arg-nitric oxide (NO) pathway. Nevertheless, some authors reported that NO inhibits [16] rather than stimulates [17,18] osteoblast proliferation. An alternative interpretation is that Arg is the precursor of ornithine, a substrate for the biosynthesis of polyamines, which are required for DNA synthesis [19].

All the amino acids, except for Trp, have enhanced the collagen synthesis at low degrees. These results are probably due to an inadequate incubation time. Actually, collagen production of osteoblasts obtained from mesenchimal stem cells increases with time [20].

The effects of the essential amino acids are probably mediated by insulin-like growth factor-I (IGF-I), which stimulates osteoblast proliferation and differentiation, type 1 collagen synthesis, osteocalcin production, and alkaline phosphatase activity [21–23]. Moreover, IGF-I is considered as an important factor for bone longitudinal growth [24] and plays a role in trabecular and cortical bone formation. Arg and Lys increase in vitro IGF-I production by osteoblast cultures [15]. IGF-I administration in osteoporotic patients enhances bone mass [25], but it is coupled with side effects probably due to non-specific effects in non-skeletal tissues. IGF-I can cause orthostatic hypotension, sinus tachycardia, bilateral parotid discomfort, weight gain and oedema [26]. Alternative approaches directed at increasing IGF-I production of osteoblasts seem to be attractive. Oral administration of Arg aspartate in the elderly enhanced serum IGF-I levels without side effects [27–29]. Therefore, it would be interesting to verify the effects of the essential amino acids on IGF-I expression and production by osteoblasts.

In summary, this preliminary work has pointed out that Arg, Lys, Met, Thr, and Trp can stimulate bone formation and could represent useful agents for the prevention and therapy of osteoporosis.

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