

Osteoblast behaviour in the presence of bisphosphonates: Ultrastructural and biochemical *in vitro* studies

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Abstract

Objective

*A positive balance in bone remodelling is an important goal of bone metabolism both in the presence of the osteoporotic processes characteristic of ageing and, especially, of prosthetic implants. The aim of the present work was to obtain new information about the initial steps of osteoblastic growth in an *in vitro* osteoblastic model in the presence of two bisphosphonates.*

Methods

Experiments were performed with Alendronate and Neridronate, two molecules used in the therapy of osteoporosis. Since differentiating features into osteoblastic cells are known to parallel the presence in the cytoplasm of alkaline phosphatase and osteocalcin, we also carried out immunohistochemical typing.

Results

Good differentiation and osteoblastic activity were generally observed in the cells in contact with these compounds, except for 10⁻⁴ Neridronate, where biochemical data clearly indicated its toxic effect on the cells.

Conclusion

The detection of osteoblastic markers associated with an ultrastructural picture of correct organellar morphology in our cultures further supports the hypothesis of a metabolically positive action of these molecules on osteoblasts.

Key words

Bisphosphonates, osteoblasts, *in vitro* study.

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Introduction

Bone undergoes remodelling throughout life. The remodelling process consists of new bone being laid down on the resorbed surface of old bone; the bone tissue thus creates an interface with itself on a continuous basis (1). Since Davies (2) reviewed several bone-cell culture systems using cell lines from different skeletal sites in different species, culture systems have emerged as a powerful means of modelling the formation of new bone on solid surfaces and producing matrix interfaces that match those found *in vivo* when new bone forms on implanted materials (1, 3).

The aim of the present study was to obtain new information about the initial steps of osteoblastic growth and extracellular-matrix deposition in an *in vitro* osteoblastic model in the presence of two bisphosphonates (Alendronate and Neridronate), well-known blockers of bone resorption whose role in osteoblastic and osteoclastic feed-back is still poorly understood (4, 5). Recent studies seem to indicate that in the presence of bisphosphonates, osteoblast activation may be involved in modulating osteoclast resorbing behaviour. On the other hand, information on the direct role of bisphosphonates on osteoblastic bone-matrix synthesis is scarce (6).

A positive balance in bone remodelling is an important goal of bone metabolism both in the presence of the osteoporotic processes characteristic of ageing and, especially, of prosthetic implants.

Materials and methods

Bisphosphonates

The effects of two bisphosphonates, Alendronate (4-amino-1-hydroxybutyridene-bisphosphonate), purchased from Merck Sharp and Dohme, and Neridronate (6-amino-1-hydroxyhexylidene-bisphosphonate), obtained from Biogen Pharma, were studied at the following concentrations: Alendronate 10^{-5} M and 10^{-7} M, Neridronate 10^{-4} M and 10^{-6} M (4).

The calculation of the doses of bisphosphonates to be used in culture media was based on the therapeutic doses usually administered to patients: Neridronate 25 mg/day intravenously or orally 50 mg/day, and Alendronate 2.5 mg/day intra-

venously or orally 5 mg/day. Since only 1% of a drug administered orally is actually adsorbed and this 1% then becomes diluted in 5 L of blood, the actual doses were: Neridronate 0.1 mg/mL (10^{-4} M) and Alendronate 0.01 mg/mL (10^{-5} M). The selection of the lower doses (Neridronate 10^{-6} M and Alendronate 10^{-7} M) was dictated by the fact that at the bone level drug absorption is much lower than at the haematic level.

Cell cultures

Osteoblasts were isolated from newborn mouse calvariae by sequential digestion for 20, 40 and 90 min at 37°C in 1 mg/mL collagenase and 0.25% trypsin. The cells of the first two digests were discarded; those released in the third digest were plated in complete DMEM supplemented with 10% foetal calf serum and penicillin/streptomycin (all from Sigma) (7). Cultures were incubated at 37°C in 5% CO₂ humidified atmosphere and used between the 6th and 8th passages. The cells were seeded at a density of 15×10^3 cells/cm² and the medium was changed twice a week. Cells were assayed and observed on days 5, 12 and 22 after the addition of the bisphosphonates because osteoblasts usually require a 2-week period to achieve complete maturation, even though important information can also be gained from culture periods of 3 to 8 days (1).

A first evaluation of cell growth was based on the degree of confluence, the semiquantitative assessment of dead cells and debris in the supernatant, and the cell's appearance. The specimens then underwent morphological, biochemical and immunohistochemical evaluation.

Morphological analysis

Ultrastructural morphological analysis was performed by transmission (TEM; Philips CM10) and scanning (SEM; Philips 505) electron microscopy. Samples were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer and post-fixed in 1% osmium tetroxide in the same buffer. They were then dehydrated in an ethanol gradient and embedded in araldite for TEM observation, or subjected to critical-point drying, mounted on aluminum stubs and gold-spattered for SEM

observation.

For SEM, cell growth in the presence of the two bisphosphonates was assessed first by image analysis (Lucia-Nikon), i.e. measuring for each sample 5 fields (magnification 500x) with an area of 1 mm², and then semiquantitatively by assigning a score: (-) no cell growth; (+) poor cell growth with marked degenerative features; (++) faint cell growth with aspects of degeneration; (+++) good cell growth with signs of degeneration; (++++) good cell growth without degenerative features.

Biochemical assays

The alkaline phosphatase (ALP) activity was measured and a total protein assay was performed on days 5, 12 and 22 of the culture. The ALP assay (Sigma Diagnostics kit) procedures for ALP depend upon the hydrolysis of p-nitrophenyl phosphate by the enzyme, yielding p-nitrophenol and inorganic phosphate. When it becomes alkaline, p-nitrophenol is converted to a yellow complex can be readily measured at 400 nm, where the colour intensity is proportional to the phosphatase activity (8). Proteins were assayed according to Lowry (9).

Immunohistochemistry

Immunohistochemical staining was performed using the avidin-biotin peroxidase complex (LASB, Dako). After 22 days in culture, cells in 4-chamber Thermanox slides were fixed for 30 min at room temperature in 95% ethanol solution and incubated with anti-mouse osteocalcin antibody (Biomedical Technologies) diluted 1:100 for 1 hr at room temperature. Control sections were processed without the primary antibody or were incubated with a reference polyclonal antibody.

After extensive washing in phosphate-buffered saline, the cells were incubated with biotinylated goat anti-mouse immunoglobulin diluted 1:20 for 30 min at room temperature. Subsequently, peroxidase-conjugated streptavidin-biotin complex (DAKO) was applied and the peroxidase activity was assessed using freshly prepared 0.05% 3,3-diaminobenzidine tetrahydrochloride (DAB) containing 0.01% H₂O₂.

Results

TEM analysis at 5 days

In the control cultures, cells displaying the features of osteoblasts were numerous in those sections obtained from the areas of cellular growth. They were large, usually rounded, with evident cytoplasmic projections that were ascribed to cell spreading, and exhibited the main features of cells involved in active protein synthesis, i.e. large nuclei with a prevalent euchromatin fraction and numerous nucleoli.

In the cytoplasm there were areas particularly rich in rough endoplasmic reticulum (RER), with dilated cisternae (Fig. 1) filled with flake-like, moderately electron-dense material, Golgi cisternae in a paranuclear position, and sacculi and vesicles of Golgi derivation. The sacculi, and in particular the vesicles, were closely associated with filaments (probably procollagen precursors) arranged in parallel and oriented towards the cell membrane. In these areas, the plasmalemma contained numerous exocytotic vesicles often fused with the membrane bilayer. To the latter, on the outer side, corresponded evident bundles of newly laid collagen cellular matrix which tended to merge into larger aggregates.

These features appeared to be present on only one of the cell sides, probably that side in contact with the substrate, while microfilaments seemed to prevail on the other side. Microfilaments were also clearly evident at the cytoplasmic periphery and close to the plasmalemma, especially in the cytoplasmic projections involved in spreading events. Cytoplas-

mic portions filled with secondary lysosomes, and mitochondria with electron-dense inclusions were observed in paranuclear position. Evident glycogen deposits, especially in the proximal areas, were also evident. Close to these large cell elements rich in cytoplasmic projections were elements of a fibroblastic nature, with large, indented nuclei and evident nucleoli which did not appear to be involved in the synthesis of fibrous matrix. In the culture there were also areas of matrix organised into undulated filaments.

In the cultures treated with 10⁻⁴ M Neridronate, there were sparse cells with evident features of degeneration, or tending to form vacuoles. Cells displayed osteoblastic features and appeared to be involved in processes of spreading, with cytoplasmic protrusions that were often thin and elongated but still had several organelles. The newly-deposited matrix was always visible in proximity of the plasmalemma - which was decorated with exocytotic vesicles - and seemed to be organised in discretely oriented bundles, larger than those observed in control cultures, which always alternated with bundles with a less ordered organisation. Fibroblast-like elements were not detected.

In the cultures treated with 10⁻⁶ M Neridronate, cells displayed the same features as in the previous culture condition. Spreading events (Fig. 2) seemed, however, to be more frequent owing to the presence of cytoplasmic projections, and more often exocytotic aspects (due to a high number of vesicles in the cytoplasm

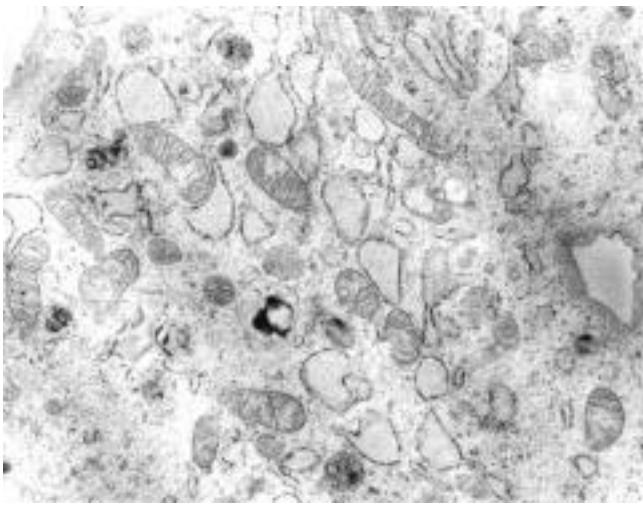


Fig. 1. Control cultures (5 days). Osteoblast cytoplasm with dilated RER cisternae and electron-dense inclusions (TEM 18, 900x).

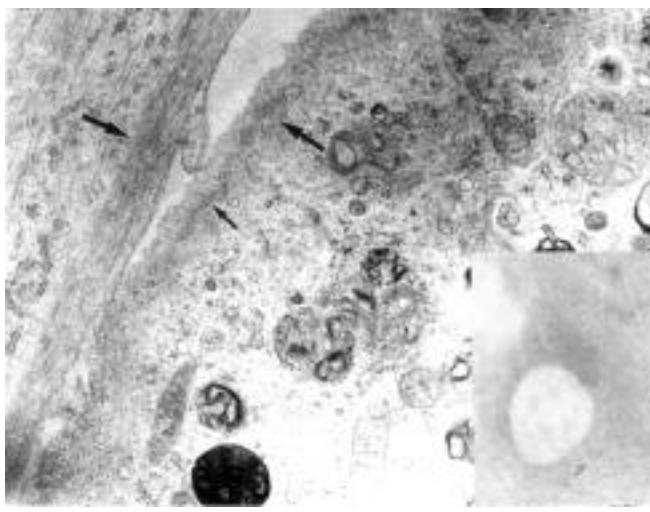


Fig. 2. 10^{-6} M Neridronate cell culture (5 days). Note the presence of numerous cytoskeletal filaments (→) in the osteoblastic cytoplasm (TEM 18, 900x). **Inset:** 22-day cultures. Osteoblast positivity for anti-osteocalcin antibody (Immunohistochemistry 400x)

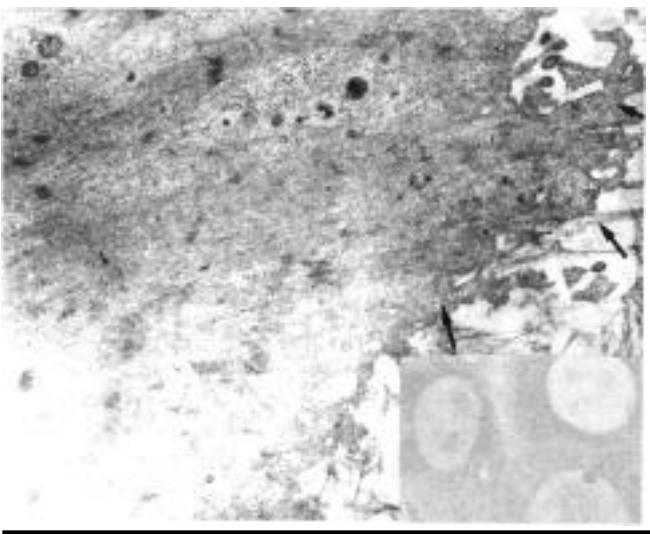


Fig. 3. 10^{-5} M Alendronate cell culture (5 days). Developed cytoskeletal network and evident features of endocytosis (→) (TEM x25,400). **Inset:** 22-day cultures. Osteoblast positivity for osteocalcin antibody (Immunohistochemistry 400x)



Fig. 4. 10^{-7} M Alendronate cell culture (5 days). Note the evident deposition of extracellular matrix in parallel with well-developed intracytoplasmic bio-energetic source (i.e., mitochondria). Some phagosomes (→) are also evident (TEM 25, 400x)

close to the plasmalemma) could be observed. There were evident cytoplasmic residual bodies in paranuclear position. The matrix fibrils appeared well oriented in the restricted areas between adjacent cells. Fibroblast-like cells were not detectable.

In the cultures treated with 10^{-5} M Alendronate, the cells exhibited on the whole the same general features as in the other experimental conditions. Cell degeneration (especially in the cytoplasmic projections), secondary lysosomes and residual bodies were occasionally ob-

served. Exocytotic vesicles were particularly numerous in the cytoplasm near the plasma membrane involved in extracellular-matrix deposition (Fig. 3). In these extracellular areas fibres were still not well organised. Again, fibroblast-like cells were not detected.

In the cultures treated with 10^{-7} M Alendronate, cells exhibited on the whole the morphological aspects of high metabolic activity. The areas of extracellular fibrous matrix deposition were at times well organised and well oriented, especially close to the osteoblastic cells (Fig. 4). These conditions coexisted with features denoting a lesser degree of matrix organisation. Areas resembling junctional contacts between cells were observed between contiguous elements. Fibroblast-like cells were not detected.

Under none of these experimental conditions did we observe areas with submicroscopic aspects compatible with mineralisation events.

TEM analysis at 12 days

The control cultures exhibited features of good preservation similar to those observed at 5 days.

The cultures treated with 10^{-4} M Neridronate were characterised by large, electron-dense elements. The cytoplasm was rich in glycogen, microfilaments and lamellar residual bodies. Extracellular-matrix deposition was not observed. In some areas, cells displayed evident features of degeneration or death.

In the cultures treated with 10^{-6} M Neridronate, zones showing cellular debris and features of cell degeneration alternated with others exhibiting a well-conserved morphology where cells rich in exocytotic cytoplasmic vesicles could be observed near the newly-formed extracellular matrix.

In the cultures treated with 10^{-5} M Alendronate, there were equal numbers of spread and polarised cells characterised by good differentiation aspects and evident, though not well-organised, extracellular-matrix deposition. Features of cell degeneration, such as mitochondrial swelling and accumulation of residual bodies were sometimes observed.

The cultures treated with 10^{-7} M Alendronate were rather less densely populated than those assayed at 5 days. Oste-

Table I. Semiquantitative evaluation of osteoblast growth in the presence of the two bisphosphonates (SEM).

	Controls		10 ⁻⁵ M Alendronate		10 ⁻⁷ M Alendronate		10 ⁻⁴ M Neridronate		10 ⁻⁶ M Neridronate	
	R	E	R	E	R	E	R	E	R	E
5 days	++±	++	++±	+	+++±	+	+++±	+	+++	
12 days	+±	+	+++	-	+++	-	±	-	++	-
22 days	+++	-	+++	-	+++	-	±	-	+++	-

R = rounded cells; E = elongated cells. Score: (-) no cell growth; (+) poor cell growth with marked degenerative features; (++) faint cell growth with aspects of degeneration; (++) good cell growth with signs of degeneration; (++++) good cell growth without degenerative features.

oblasts appeared to be in good morphological and proliferative condition. The synthesis of filamentous extracellular matrix was fairly good and the matrix was better organised and oriented than in cells cultured with 10⁻⁵ M Alendronate. Several fusiform, elongated elements bearing features of migrating osteoblasts were also observed.

SEM analysis

The semiquantitative evaluation of cell growth is summarised in Table I and Figure 5. At 5 days, nearly all samples displayed good cell growth with generally rounded or, occasionally, elongated osteoblast-like cells. In some areas the cells

were very densely packed. At 12 days proliferation was still good, except for the cells cultured with 10⁻⁴ M Neridronate, where elements appeared to be severely damaged and exhibited features of cytoplasm fragmentation. Similar features were observed at 22 days, including the degeneration observed with 10⁻⁴ M Neridronate.

Biochemical assays

The results of the biochemical assays are reported in Table II.

At 5 and 12 days there were no differences between the bisphosphonate-treated samples and controls in terms of ALP activity. At 22 days, in the cultures

treated with 10⁻⁵ M Alendronate, ALP activity was higher compared with the control and all other treated, viable samples. The good ALP activity also detected in Neridronate 10⁻⁶ M cultures suggests a good bio-energetic status at least at the level of the single well-preserved osteoblast. The high ALP activity values detected in the samples treated with 10⁻⁴ M Neridronate at 12 and 22 days were not reliable as in these cases their protein content fell outside the linearity range.

Based on the data from the morphometrical analysis, ALP activity could be correlated with the number of cells per well (Fig. 6).

Immunohistochemistry

The osteoblastic nature of the cells was confirmed by the presence of osteocalcin. The level of expression of this cell marker appeared to be similar under all culture conditions and was not closely related to the pharmacological treatment (Figs. 2 and 3).

Discussion

Bisphosphonates have several clinical applications in diseases associated with

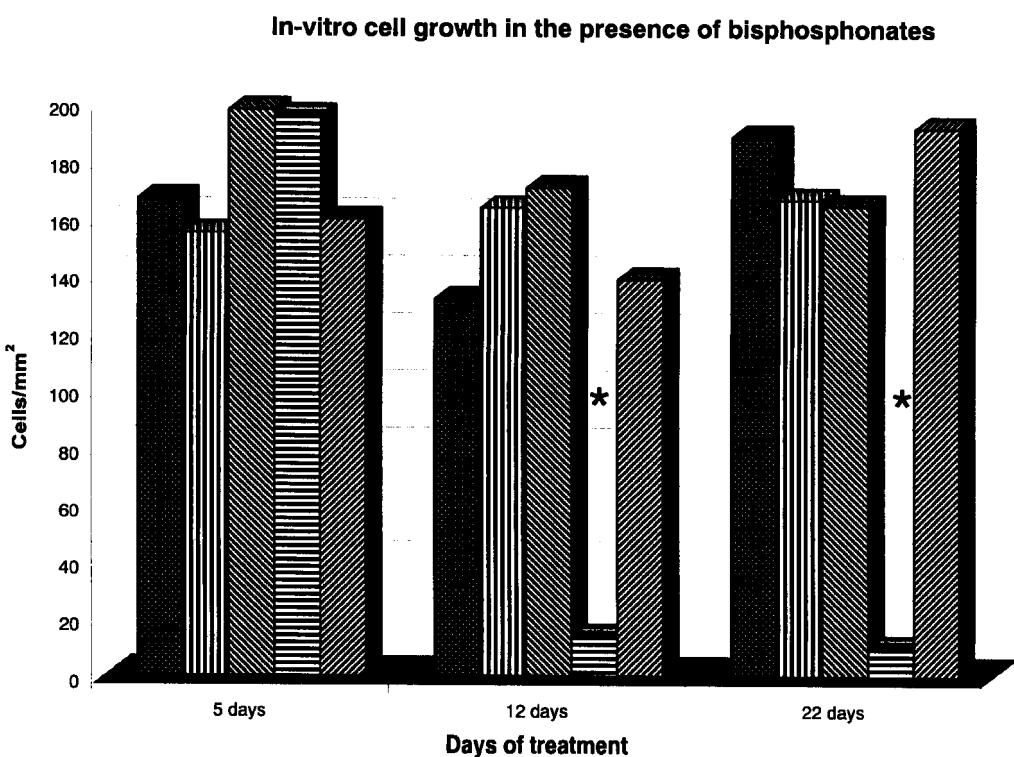


Fig. 5. Histogram of *in vitro* cell growth in the presence of the two bisphosphonates. Mean value are reported (SD = ± 5%). *Significantly different from the control.

Table II. Effects of treatments with two bisphosphonates on ALP expression in newborn-mouse osteoblasts.

Treatment	ALP activity (S.U./mg protein \pm)
5 days	
Control	6.35 \pm 1.42
10 ⁻⁵ M Alendronate	5.82 \pm 1.74
10 ⁻⁷ M Alendronate	6.69 \pm 2.45
10 ⁻⁴ M Neridronate	5.71 \pm 1.18
10 ⁻⁶ M Neridronate	5.80 \pm 1.08
12 days	
Control	5.09 \pm 0.61
10 ⁻⁵ M Alendronate	5.70 \pm 2.23
10 ⁻⁷ M Alendronate	5.33 \pm 0.39
10 ⁻⁴ M Neridronate	12.60 \pm 0.12*
10 ⁻⁶ M Neridronate	5.47 \pm 0.85
22 days	
Control	6.03 \pm 0.92
10 ⁻⁵ M Alendronate	8.04 \pm 1.93
10 ⁻⁷ M Alendronate	5.78 \pm 0.78
10 ⁻⁴ M Neridronate	129.42 \pm 3.99*
10 ⁻⁶ M Neridronate	6.74 \pm 1.10

Data represent the mean \pm standard deviation of quadruplicate determinations.

§ S.U. = Sigma Units. One S.U. of phosphatase activity is defined as the amount of enzyme activity that will release 1 μ mol of p-nitrophenol per hour under test conditions described by Bissey *et al.* (9).

* Not reliable; toxic effect.

increased bone turnover, and in addition they inhibit osteoclast-mediated bone resorption by direct and indirect actions on osteoblasts. Bisphosphonates are the treatment of choice in Paget's syndrome, reducing hypercalcemia associated with disease. In patients with post-menopausal osteoporosis they prevent further bone loss and reduce pain and the incidence of fractures (10).

Bone mineralisation defects which did, occasionally, occur with first-generation bisphosphonates, do not appear to arise with the new ones, among which Etendronate and Alendronate are those more frequently utilised.

Recent pharmacological studies on bone-marrow cultures show, in agreement with our data, that these bisphosphonates exert a positive influence on mesenchymal progenitor-cell metabolism (11). These pharmacologically active large molecules could come between the extracellular environment and the membrane integrins, influencing the modulation of the osteoblastic cell cycle and enhancing the transition towards greater osteoblast differentiation (5). The bisphosphonates would thus act by attaching themselves mainly to the osteoclast surface, preventing the degradation of the

calcified tissue (12), and could also act as osteoblast kinases. This behaviour can partially be attributed to the interaction of the cellular matrix with the membrane's integrins, which mediate the adhesion between the cell membrane and extracellular signals (13). Therefore, integrins are believed to induce kinase activation in "focal adhesions", triggering the autophosphorylation of their endocellular tyrosine residues, but also of other endocellular molecules (10, 14). In our study, the biochemical analysis of alkaline phosphatase activity showed consistent synthesis of this enzyme in the presence of the two bisphosphonates. Good differentiation and osteoblastic activity were generally observed in the cells in contact with these compounds, except for 10⁻⁴ M Neridronate at 12 and 22 days, where biochemical data clearly indicate its toxic effect on the cells. The good ALP activity correlated to the number of cells per well detected with the two bisphosphonates indicate the positive metabolic interference at a dose which, transported to *in vivo* therapeutic administration, correspond to the drug's concentration in bone. Our data thus show good compatibility between osteoblasts and bisphospho-

ALP Activity/Cells per well

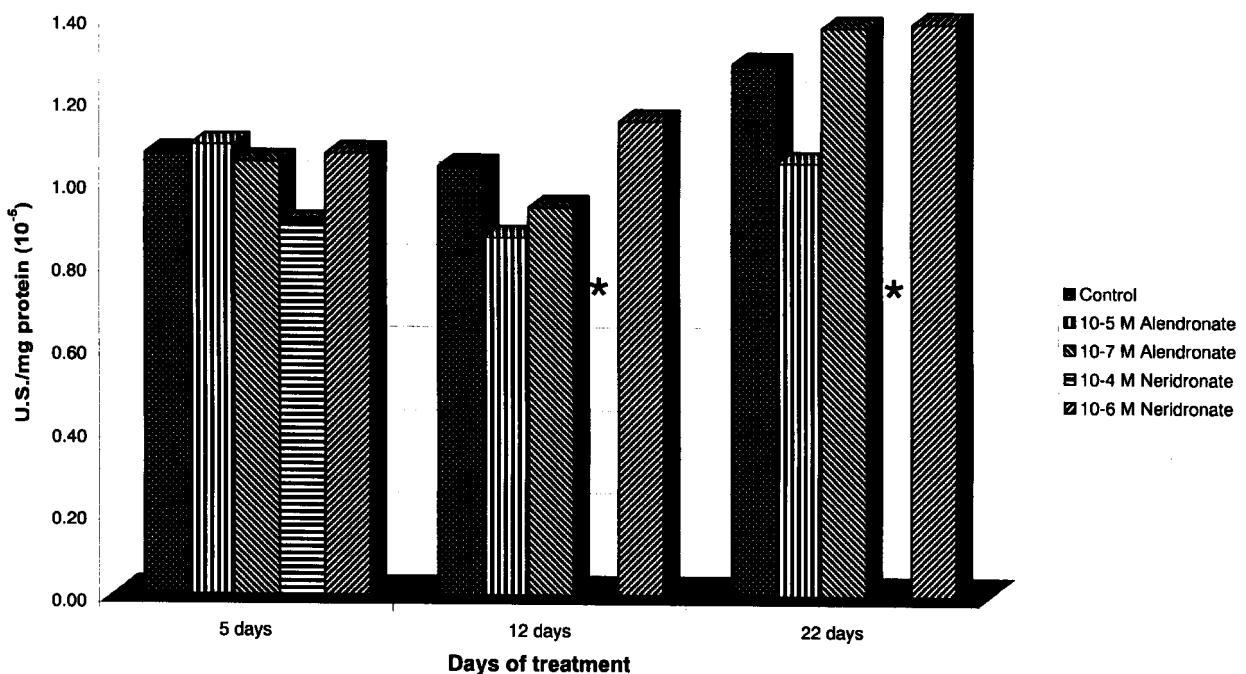


Fig. 6. Alkaline phosphatase (ALP) activity per cells; *ALP activity not evaluated as the protein content falls outside the linearity range.

nates at the therapeutically active lower tested doses. Since in osteoblasts the differentiating features parallel the presence of alkaline phosphatase and osteocalcin in the cytoplasm (12), the detection in our cultures of these markers associated with an ultrastructural picture of correct organellar morphology support the hypothesis of a metabolically positive action of bisphosphonates on osteoblasts. Therefore, the utilisation of these anti-resorption compounds at doses comparable to those administered therapeutically to osteoporotic patients could provide bio-energetically positive stimuli to these osteoblastic cell populations (4). We intend to continue these studies by applying the same methodologies to osteoblasts from osteoporotic subjects, where some metabolic differences can be observed compared with those from non-osteoporotic ones (15). This appears to be a suitable means to study the bisphosphonates *in vitro* without losing sight of the fact that human biology must be studied as the biology of the single affected individual rather than as a disorder in its "statistical" dimension.

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