INTRODUCTION:
Bisphosphonates (BPs) are potent inhibitors of osteoclastic bone resorption, widely used in the treatment of osteoporosis, Paget’s disease, hypercalcemia of malignancy, multiple myeloma, and bone metastases associated with breast, prostate, lung, and other soft tissue tumors. Osteonecrosis of the jaws (ONJ) is a recently described clinical condition that has been associated with the use of nitrogen-containing bisphosphonates, including pamidronate. Previous in vitro studies using the osteogenic cell lines and primary cells isolated from the long bones suggested possible anabolic effects of BPs on bone formation (increased cell proliferation, expression of bone markers and mineralization) at low concentrations (10^{-5} - 10^{-10} M), whereas at physiologically relevant higher concentrations (10^{-4} M) inhibition of cell growth and induction of apoptosis were noted (1-4). Craniofacial bones differ from long bones in their developmental lineage and the process of bone formation, therefore the aim of our study was to specifically evaluate the effects of pamidronate on human primary osteoblasts isolated from samples of alveolar and long bone tissue.

METHODS:
Primary human osteoblast-like cells (“osteoblasts”) were isolated from discarded alveolar (n=6) and long bone surgical procedures (n=3). All protocols were approved by the Columbia University IRB. Bone tissue was minced, washed and cultured in DMEM/F12 with 20% FBS, gentamicin and fungizone. Proliferation of first and third passage cells was compared between the two bone sources (CellTiter96®Assay, Promega). The effect of pamidronate (Sigma) on cell proliferation was evaluated at 24, 48, 72 and 168hrs after the addition (10^{-5} - 10^{-10} M) to cultures. Alkaline phosphatase (AP) activity was evaluated by AP activity stain at 24 and 168hrs qualitatively (fast-blue salt staining, Sigma) and quantitatively (conversion of p-nitrophenyl phosphate to p-nitrophenol, Sigma) in the control medium (DMEM/F12 with 10% FBS, gentamicin and fungizone) and osteogenic medium (control medium with 10 mM β-glycerophosphate, 100 nM dexamethasone and 0.05 mM L-ascorbic acid 2-phosphate). Induction of apoptosis was evaluated at 24, 48 and 72hrs by Tunel assay (Roche). Statistical significance was assessed by one-way ANOVA analysis (IMP software, SAS Institute).

RESULTS:
Primary human osteoblasts isolated from the alveolar bone exhibited a higher level of proliferation (average 2-3 doublings, n=6) compared to long bone osteoblasts (1±1 doubling, n=2) during the first passage. Alveolar osteoblasts showed similar proliferation in the third passage, whereas the long bone osteoblasts demonstrated a higher level of proliferation (aver age 2-3 doublings, n=6) compared to all other groups (1±1.4%) during the first passage. AP activity stain was positive in cultures supplemented with 10^{-4} M pamidronate (~0.9 and ~0.3 nmol nitrophenol/(min·well) in samples 1 and 2). AP activity of these groups was significantly higher compared to the osteogenic groups treated with 6x10^{-5} M and 10^{-4} M pamidronate (~0.3 and ~0.1 nmol p-nitrophenol/(min·well) in samples 1 and 2) (p<0.05). Tunel assays showed significant increases in the number of apoptotic cells (17±8%) at 10^{-4} M pamidronate compared to 6x10^{-5} M, 10^{-4} M and 0 M groups (1±1.4%) at 72hrs after treatment (Figure 1C, p=0.001, n=3). In addition, DAPI nuclear staining showed condensation, and the cells exhibited a stressed morphology at 48hrs, and started detaching at 72hrs.

DISCUSSION:
Our experiments indicate differences in the proliferative potential of primary human osteoblasts isolated from the alveolar and long bone samples. Similar differences were reported for marrow stromal populations isolated from the orofacial and long bones (5). Significant decreases in alveolar osteoblast proliferation and viability were observed at the highest pamidronate concentration (10^{-4} M). A similar inhibitory effect was observed previously in immortalized human osteoblast (2) and primary mouse calvarial osteoblast cultures (5). Our results were highly consistent between 4 different bone samples, and contrast with several of the previous reports, as we have not observed significant anabolic effects of pamidronate on osteoblasts (based on proliferation data and AP activity assessments) (1-4). Our data indicate that the observed pamidronate toxicity at concentrations >6x10^{-5} M may in part result from the induction of apoptosis, which was noted previously at similar concentrations and treatment duration in mouse calvarial osteoblast cultures (6). Interestingly, in our previous experiments with oral mucosa cells decreased cell viability and inhibition of wound healing was observed at slightly higher pamidronate concentrations, but no apoptosis induction was noted (7). Our future work will be aimed at studying the specific mechanisms of bisphosphonate toxicity in alveolar osteoblasts and primary osteoblast/osteoprogenitor populations isolated from the human long bones.

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REFERENCES:

Figure 1: Effects of pamidronate on proliferation (A), viability (B), ALP activity (C), and induction of apoptosis (D) of human alveolar osteoblasts. Data represent average ± SD of 3-4 samples. * indicates significant difference to all other groups, # indicates significant difference to 24hrs, dotted lines indicate cell responses.