INTRODUCTION
Caffeine is one of the most commonly consumed pharmacologically active compound in the world, certainly in Europe and North America. Caffeine-containing beverage consumption has been reported to be associated with reduced bone mass and increased fracture risk in some observational studies. Caffeine consumption has been reported to decrease bone mineral density (Barrett-Connor et al. 1994), increase the risk of hip fracture, and negatively influence calcium retention (Meyer et al. 1997). However, the role of caffeine as a risk factor for bone loss is still controversial. In this study, we investigated the influence of caffeine on in vitro osteoblasts metabolism. The biocompatibility has been evaluated by means of cytototoxicity and cyto-compatibility tests. Cell proliferation as well as the expression of some biochemical parameters of osteoblastic phenotypes have been monitored, the effect of caffeine on in vitro osteoblasts metabolism. The results of this study will provide new information for understanding the influence of caffeine on osteoblasts metabolism and the possible mechanisms of osteoblasts apoptosis.

MATERIALS AND METHODS
Newborn Wistar-rat calvaria osteoblasts were used in this study. In the first part of this study, the effects of various concentrations of caffeine on bone cell activities were evaluated by using MTT assay. Seven different concentrations (100, 50, 10, 5, 1, 0.5, 0.1 mM) were tested for 1 day, 3 days, 7 days and 14 days period. 10mM caffeine solution was selected for further study. Then, a concentration of 1 x 10^6 cells/100 μl was added to 35 mm wells of a 6-well plate. After 48 hours, the media were changed and the cells were incubated in α-MEM with 10% fetal calf serum, antibiotics, L-ascorbic acid (50 μg/ml), 5 mM β-glycerophosphate and 10-8 M dexamethasone. The day of changing specific medium was day zero. From day zero of culture, 10mM caffeine solution was added. The medium was changed every 3–4 days; alkaline phosphatase (ALP) staining, von Kossa stain for mineralized nodules and biochemical parameters including alkaline phosphatase, lactate dehydrogenase, prostaglandin E2 and total protein were performed at day 1, 3, and 7. For the DNA fragmentation, a concentration of 1 x 10^6 cells/100 μl was added to 90 mm disc; six different concentrations of caffeine solution (0, 0.5, 1.0, 2.5, 5.0, 10.0 mM) were tested. The medium was changed every 3–4 days; the DNA fragmentation analysis was performed at day 1, 3, and 7.

RESULTS
When osteoblast cells cultured with caffeine for one day, there was no statistically significant change in the formation of formazan; while at the 3rd day’s culture, decreased osteoblasts activities were observed in the presence of various concentrations of caffeine. We selected the 10 mM concentration of caffeine for the further biochemical study because the osteoblasts showed highest activities during the 3rd and 7th testing period. The formation of ALP positive staining colonies and mineralization nodules formation in the osteoblast cultures were significantly affected by caffeine. In control samples, the osteoblasts differentiated as the cultured period increased. When osteoblasts cultured with 10 mM caffeine, the viability of osteoblasts was decreased and the residual cells lost their reaction to ALP stain and similar results were observed on the von-Kossa staining. Corresponding to the viability of osteoblasts was decreased significantly in the presence of 10 mM of caffeine, the intracellular LDH and ALP content decreased significantly and the LDH secreted into the medium increased significantly. Both the intracellular PGE2 and the PGE2 secreted into medium decreased significantly at the 3rd and 7th day’s culture. At the same time, total protein contents were relatively preserved. Activation of an irreversible commitment to cell death by caffeine was clearly demonstrated in the DNA fragmentation analysis. The formation of DNA fragments was easily observed when osteoblasts cultured with caffeine. Electrophoresis of genomic DNA from osteoblasts that were exposed to 5.0 and 10 mM caffeine showed the characteristic laddering pattern (in the size of 200 – 1000 bp) that leads to cell death in the first day’s culture; while in the concentrations of 0.5, 1.0 or 2.5 mM caffeine, the appearance of DNA fragmentation appeared at the 3rd day’s culture with the characteristic laddering pattern in the size of 200 – 1000 bp.

DISCUSSION
Coffee is one of the most widely consumed psychoactive beverages throughout the world. Many investigators have demonstrated that caffeine, one of the main constituents of coffee, has a variety of pharmacological and cellular responses in a wide spectrum of biological systems. The effects of coffee on bone metabolism are still controversial, although several studies have suggested that caffeine and/or heavy coffee consumption is associated with a significant increase in risk of fracture, osteoporosis, and periodontal disease (Kamagata-Kiyoura et al. 1999). Our results suggest that caffeine has potential deleterious effect on the osteoblasts viability, which may enhance the rate of osteoblasts apoptosis. The caffeine-induced osteoblasts apoptosis probably is one of the major factors in the caffeine-ingestion related osteoporosis observed in clinical medicine.

REFERENCES

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