# Effects of Hydrochlorothiazide and Furosemide Diuretics on Human Bone Marrow Stromal Osteoprogenitor Cells

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**Thiazide diuretics have been shown to decrease bone loss and improve bone mineral density, while long-term furosemide therapy has been suggested to decrease bone mineral content. However, the direct effects of these diuretics on osteoblastic cells are not well established. Some investigators have reported direct effects of thiazides on osteoblastic cells but the results remain controversial, and there are few data about the direct effect of furosemide on osteoblastic cells. We investigated the effects of hydrochlorothiazide (HCTZ) and furosemide on proliferation, alkaline phosphatase activity, osteocalcin, and interleukin-6/interleukin-11 (IL-6/IL-11) secretion in cultured normal human bone marrow stromal osteoprogenitor cells (hBMSCs). Treatment with HCTZ or furosemide for 24 hours in the concentration range of $10^{-6}$ to $10^{-4}$ mol/L did not affect $^{3}$H-thymidine incorporation in hBMSCs. Cellular alkaline phosphatase activity and osteocalcin production were not changed significantly by treatment with HCTZ or furosemide (up to $10^{-4}$ mol/L) during culture. There was also no significant difference in IL-6 and IL-11 production in hBMSCs. These results suggested that HCTZ or furosemide had no significant direct effect on proliferation, alkaline phosphatase activity, osteocalcin, and IL-6/IL-11 production in hBMSCs, and the effects of these diuretics on bone mass may be related to the indirect action on calcium balance.**


THIAZIDE DIURETICS have been shown to decrease the rate of bone loss and to improve bone mineral density in patients treated with such medication.[1,2] Lemann et al[3] reported that hydrochlorothiazide (HCTZ) inhibited bone resorption, and a longitudinal study also showed a decrease in the rate of bone loss with thiazides.[4] In addition, long-term use of thiazides also reduces the prevalence of bone fractures in aged patients.[5-7] In contrast, long-term furosemide therapy was reportedly associated with decreased bone mineral content in preterm infants,[8] and may contribute to the reduced bone density of patients with congestive heart failure and cardiac transplant recipients.[9] These contrasting effects on bone of the 2 commonly used diuretics have been attributed to the different effects of these diuretics on urinary calcium excretion and calcium balance: thiazides are known to conserve calcium,[10,11] but furosemide wastes it.[12,13]

Recently, direct effects of thiazides on osteoblastic cells have been suggested but remain controversial. Some groups reported that HCTZ increased $^{3}$H-thymidine incorporation in human primary osteoblasts,[14] whereas others reported that it exerted no effect on DNA synthesis in rat osteosarcoma UMR cells.[15] Aubin et al[16] reported that thiazide diuretics inhibited osteocalcin secretion and macrophage colony-stimulating factor (M-CSF) production by the MG-63 human osteoblast-like cell line. However, there are few data about the direct effects of thiazides or furosemide on normal human osteoblastic cells. Previous studies have shown that bone marrow stromal cells are fibroblast-like cells that include precursors for osteoblasts,[17] and isolated bone marrow stromal cells are able to produce cartilage and bone within a diffusion chamber implanted in an animal.[18] In the present study, we investigated the effects of HCTZ and furosemide on proliferation, activity, and secretion of interleukin-6 (IL-6) and IL-11, which are known to be important mediators of bone resorption, in cultured normal human bone marrow stromal osteoprogenitor cells (hBMSCs).

## MATERIALS AND METHODS

### *hBMSC Culture*

hBMSCs were isolated from ribs that were discarded at the time of open thoracotomy in patients without metabolic bone disease as described previously.[19,20] Briefly, the ribs were excised aseptically, cleaned of soft tissue, and opened longitudinally. The exposed bone marrow was flushed out using several washes of serum-free α-minimum essential medium ([α-MEM], Sigma, St Louis, MO) and centrifuged at 1,400 rpm for 10 minutes. Cell pellets were resuspended in culture medium, and the hBMSC fraction was obtained by Ficoll/Hypaque (specific gravity 1.077; Nycomed, Oslo, Norway) gradient centrifugation. The cells were seeded into a 75-cm$^2$ plastic culture flask at a density of $4 \times 10^5$ cells/cm$^2$ and cultured in α-MEM containing 10% fetal bovine serum ([FBS] GIBCO, Grand Island, NY), penicillin, and streptomycin (100 U/mL and 100 μg/mL, respectively; Sigma). The medium was changed twice weekly from the second week, and when the cells were grown to 80% confluence, they were subcultured using 0.01% trypsin and 0.05% EDTA. The second-passage cells were used in the experiments.

Previous studies[21,22] have shown that when cultured to confluence in the presence of serum, these cells possess many of the phenotypic characteristics of differentiated osteoblasts, including production of type I procollagen and formation of bone nodules. We also confirmed that these cells deposited calcium into the extracellular matrix and expressed mRNAs characteristic of osteoblastic cells such as alkaline phosphatase, α1(I)collagen, and osteopontin.[23] The absence of monocytic cells was confirmed by staining cultures for nonspecific esterase.[24]

### *$^{3}$H-Thymidine Uptake*

Cell proliferation was assessed by measurement of $^{3}$H-thymidine incorporation. hBMSCs were seeded into 24-well plates at a density of $2.0 \times 10^4$/well with α-MEM containing 10% FBS and penicillin-streptomycin. The cells were treated with HCTZ or furosemide at a concentration of $10^{-6}$ to $10^{-4}$ mol/L for 24 hours. HCTZ and furosemide were dissolved in $10^{-2}$ mol/L NaOH, and an initial $10^{-2}$ mol/L stock solution was serially diluted at concentrations of $10^{-4}$ to

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*Submitted November 6, 1998; accepted July 25, 1999.*

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*0026-0495/00/4901-0015$10.00/0*

$10^{-6}$ mol/L. The vehicle was added to the control cultures, and the final concentration of NaOH in culture media did not exceed $10^{-6}$ mol/L and the pH of the media did not exceed 7.6. $^{3}$H-thymidine (1.0 μCi/mL; New England Nuclear, Boston, MA) was added for the last 3 hours of the incubation period. $^{3}$H-thymidine incorporation into trichloroacetic acid–precipitable material was measured by standard methods.[25] The number of cells in each well was determined by a quadruplicate hemocytometer count of trypsin-EDTA–released cells at the end of the culture period, and the measured activities of incorporated $^{3}$H-thymidine were corrected with the cell counts.

### *Alkaline Phosphatase Activity*

Cells were seeded into 12-well plates at a density of $4 \times 10^{4}$/well and cultured for 2 days in α-MEM containing 5% FBS, and then $10^{-6}$ to $10^{-4}$ mol/L HCTZ or furosemide was added. Vehicle- and dexamethasone ($10^{-7}$ mol/L)-treated wells were used as negative and positive controls, respectively. After 72 hours of additional culture, the medium was removed and the cell layer was washed and assayed for alkaline phosphatase activity by measuring *p*-nitrophenyl phosphate hydrolysis.[26] The total amount of protein in the cell layer was determined by the Lowry method, and the measured alkaline phosphatase activity was corrected with the protein content.

### *Osteocalcin Secretion*

Cells were seeded into 12-well plates at a density of $4 \times 10^{4}$/well and cultured for 2 days in α-MEM containing 0.1% bovine serum albumin, 50 μg/mL ascorbic acid, and 50 nmol/L $1,25(OH)_2D_3$, and then $10^{-6}$ to $10^{-4}$ mol/L HCTZ or furosemide was added. Vehicle- or dexamethasone ($10^{-7}$ mol/L)-treated wells were used as controls. After 72 hours of additional culture, the conditioned media were collected, centrifuged free of cellular debris, and stored at −20°C until needed for assay. The concentration of osteocalcin in the media was measured by radioimmunoassay using a commercial kit (Metra Biosystems, Mountain View, CA), and the data were normalized to the total cellular protein content.

### *IL-6 and IL-11 Production*

The cells were subcultured in 48-well plates ($3 \times 10^{4}$/well) containing α-MEM and 5% charcoal–stripped serum for 2 days. Subsequently, the media were substituted with fresh media containing various doses of HCTZ or furosemide ($10^{-4}$ to $10^{-6}$ mol/L) and then cultured for an additional 72 hours. IL-1α (500 U/mL)- and dexamethasone ($10^{-7}$ mol/L)-treated wells were used as positive and negative controls, respectively. After 72 hours, the conditioned media were collected, centrifuged free of cellular debris, and stored at −20°C until IL-6 and IL-11 assay. IL-6 and IL-11 concentrations in the media were measured by enzyme-linked immunosorbent assay (ELISA) methods using commercial kits (IL-6 kit from Genzyme, Cambridge, MA; IL-11 kit from R&D Systems, Minneapolis, MN). The data are expressed as the amount of IL-6 or IL-11 produced per $10^{5}$ cells.

### *Statistics*

All of the experiments were repeated at least 3 times using different hBMSC preparations, and representative data are shown in the figures. The significance of differences between treatment groups was assessed using the Mann-Whitney *U* test or ANOVA and post hoc analysis with Duncan's multiple-range test as appropriate.

## RESULTS

### *Effects of HCTZ and Furosemide on Proliferation of hBMSCs*

Treatment with HCTZ for 24 hours at the concentration range of $10^{-6}$ to $10^{-4}$ mol/L did not affect $^{3}$H-thymidine incorporation (525 ± 58 cpm/$10^{4}$ cells in $10^{-4}$ mol/L HCTZ *v* 620 ± 68 cpm/$10^{4}$ cells in the control; Fig 1). There was also no significant difference between furosemide ($10^{-4}$ mol/L)-treated cells and control cells (635 ± 75 *v* 620 ± 68 cpm/$10^{4}$ cells).

### *Cellular Alkaline Phosphatase Activity and Osteocalcin Production*

Cellular alkaline phosphatase activity was similar in HCTZ ($10^{-4}$ mol/L)-treated cells (280 ± 23 nmol/mg protein/min) and furosemide ($10^{-4}$ mol/L)-treated cells (278 ± 15 nmol/mg protein/min) compared with vehicle-treated cells (251 ± 27 nmol/mg protein/min; Fig 2). Treatment with HCTZ or furosemide for 72 hours also did not change osteocalcin production significantly (2.39 ± 0.27 ng/mg protein in $10^{-4}$ mol/L HCTZ *v* 2.55 ± 0.34 in furosemide *v* 2.81 ± 0.24 in vehicle; Fig 3).

### *IL-6/IL-11 Production*

There was no significant difference in IL-6 production between HCTZ ($10^{-4}$ mol/L)-treated (786 ± 134 pg/$10^{5}$ cells) or furosemide ($10^{-4}$ mol/L)-treated cells (742 ± 113 pg/$10^{5}$ cells) and vehicle-treated cells (877 ± 62 pg/$10^{5}$ cells; Fig 4). IL-11 production in hBMSCs was also similar in all 3 groups

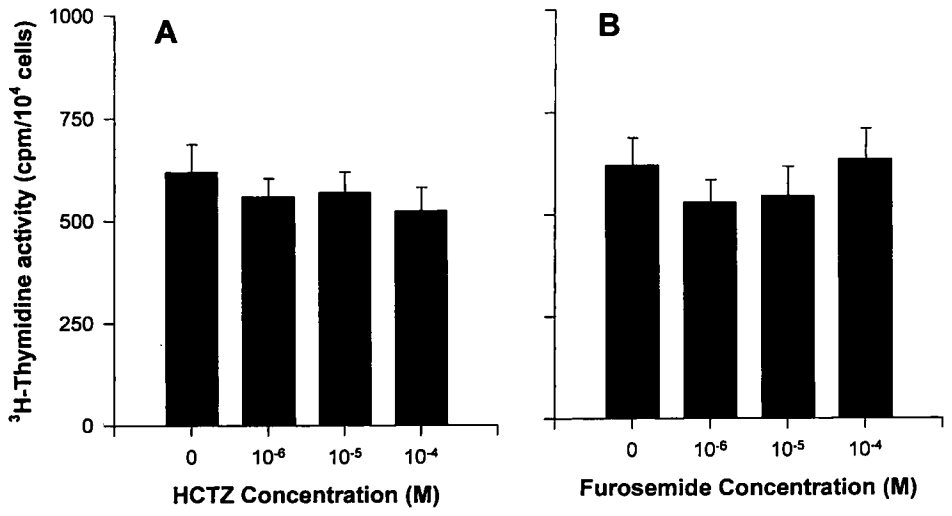


**Fig 1. Effects of HCTZ (A) and furosemide (B) on $^{3}$H-thymidine uptake in hBMSCs. Cells in each well were treated with HCTZ or furosemide for 24 hours, and $^{3}$H-thymidine (1.0 μCi/mL) added for the final 3 hours. Values are mean ± SD of 8 determinations.**

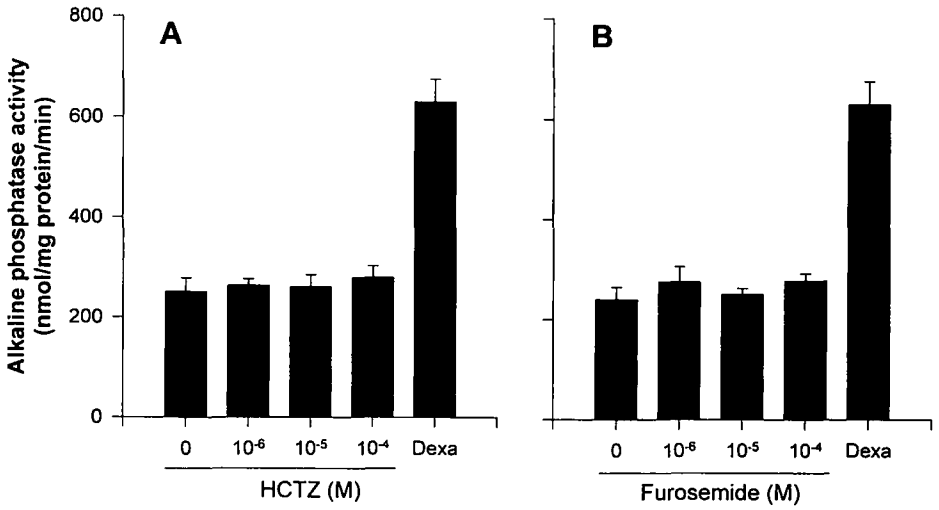


**Fig 2. Effects of HCTZ (A) and furosemide (B) on cellular alkaline phosphatase activity in hBMSCs. Data were normalized to total protein content of the cell layer. Dexamethasone ($10^{-7}$ mol/L)-treated wells (Dexa) were used as a positive control. Values are mean ± SD of 6 determinations.**

(233 ± 18 pg/$10^5$ cells in $10^{-4}$ mol/L HCTZ *v* 248 ± 28 in furosemide *v* 286 ± 17 in vehicle; Fig 5).

## DISCUSSION

In the present study, we found that HCTZ did not affect proliferation, differentiated activity such as alkaline phosphatase activity and osteocalcin production, or IL-6/IL-11 secretion in primary cultured normal hBMSCs. Although a number of clinical studies have shown positive effects of thiazide diuretics on bone, the exact mechanism has not been elucidated. Thiazide diuretics have been known to increase calcium reabsorption in renal tubules and to decrease urinary calcium excretion, resulting in positive calcium balance.[10,11] In addition to such indirect effects, some investigators suggested that thiazides have direct effects on osteoblastic cells. For instance, Song and Wergedal[14] reported that HCTZ increased $^3$H-thymidine incorporation in human primary osteoblasts. In contrast, Hall and Schaueblin[15] reported that thiazide had no effect on DNA synthesis in rat osteosarcoma UMR cells. Barry et al[27] provided evidence for direct effects of thiazide diuretics on UMR-106 cells by showing that thiazide inhibited sodium-chloride cotransporter activity, thereby altering intracellular calcium regulation in these cells. Recently, Aubin et al[16] reported that thiazide diuretics inhibited osteocalcin secretion and M-CSF production by the MG-63 human osteoblast-like cell line. The causes of such discrepancies among previous studies are not clear at present, but may be due to the differences in the species of cell origin, the developmental stage of the cells, or the culture systems. In this regard, it is notable that most of the previous results were from experiments using animal cells or transformed cells, except for the study by Song and Wergedal.[14] To our knowledge, the present study is the first to examine the direct effects of thiazide and furosemide using normal hBMSCs.

IL-6 and IL-11 have been proposed as the main mediators responsible for the enhanced osteoclast formation and bone loss observed in a variety of clinical conditions.[28,29] IL-6 and IL-11 production by osteoblasts and bone marrow stromal cells is stimulated by various systemic and local factors including PTH and IL-1, and they in turn stimulate the early stage of osteoclastogenesis. We examined the possible effects of thiazide and furosemide on IL-6/IL-11 production in hBMSCs, but we did not observe any significant effect of these diuretics on the production of these cytokines.

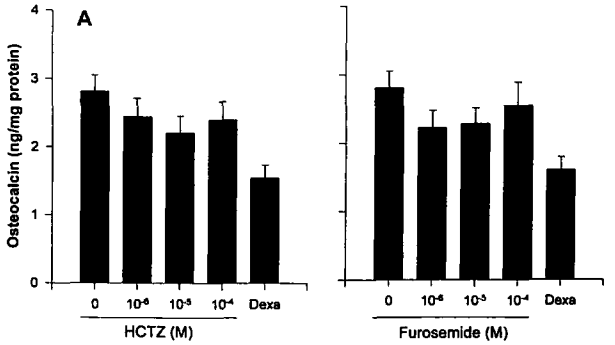


**Fig 3. Effects of HCTZ (A) and furosemide (B) on osteocalcin production in hBMSCs. Osteocalcin secretion in media for 72 hours was determined by radioimmunoassay and normalized to total protein content of the cell layer in each well. Dexamethasone ($10^{-7}$ mol/L)-treated wells (Dexa) were used as a negative control. Values are mean ± SD of 6 determinations.**

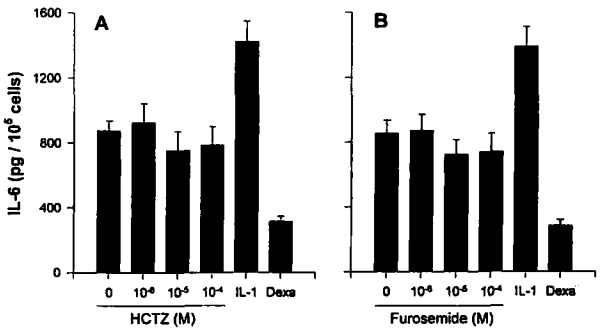


**Fig 4. Effects of HCTZ (A) and furosemide (B) on IL-6 production in hBMSCs. IL-6 secretion in media for 72 hours was measured by ELISA and normalized to the number of cells in each well at the end of incubation. IL-1α (500 U/mL)- and dexamethasone ($10^{-7}$ mol/L)-treated wells (Dexa) were used as positive and negative controls, respectively. Values are mean ± SD of 8 determinations.**

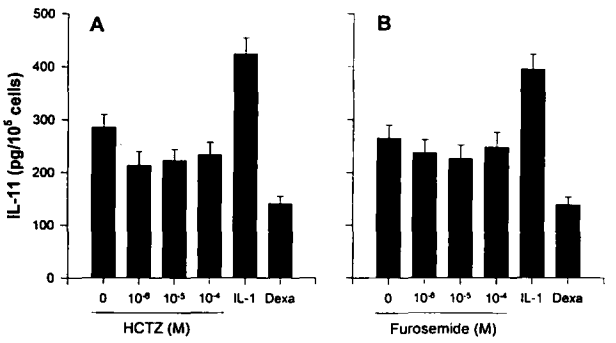


**Fig 5. Effects of HCTZ (A) and furosemide (B) on IL-11 production in hBMSCs. IL-11 secretion in media for 72 hours was measured by ELISA and normalized to the number of cells in each well at the end of incubation. IL-1α (500 U/mL)- and dexamethasone ($10^{-7}$ mol/L)-treated wells (Dexa) were used as positive and negative controls, respectively. Values are mean ± SD of 8 determinations.**

Our results are consistent with the previous idea that the positive effect of thiazides on bone mass could be the result of an indirect effect on calcium balance. However, there is another possibility that thiazide may exert a protective effect on bone via a direct action on osteoclastic cells. In line with this possibility, Hall and Schaueblin[15] reported that HCTZ at a concentration range of $10^{-6}$ to $10^{-4}$ mol/L dose-dependently inhibited bone resorption by isolated rat osteoclasts in a bone slice assay. They suggested that the in vitro effect of HCTZ on bone resorption might be due to inhibition of osteoclast carbonic anhydrase. Further studies are needed to examine the direct effects of thiazides on human osteoclastic cells and bone resorption.

It is well known that furosemide can increase urinary calcium excretion with both short- and long-term administration. It has been suggested that the renal calcium wastage causes secondary hyperparathyroidism,[30-32] which may lead to increased bone loss.[8,33] However, Koo et al[34] observed that long-term furosemide therapy led to growth failure and decreased bone mineral in newborn rats, but there was no significant difference with respect to serum alkaline phosphatase and PTH concentrations, thereby failing to support the presence of secondary hyperparathyroidism. This suggested that another mechanism might be involved in the furosemide-induced bone loss, but few studies have reported on the direct effects of furosemide on osteoblastic cells. From our results, it is conceivable that furosemide has no significant direct effect on the proliferation or activities of osteoblastic cells, consistent with the previous notion that the effect of furosemide on bone is also indirect.

However, it should be noted that our conclusion is not definitive, because there are several limitations of this study. First, there are many other osteoblastic functions that were not determined. In addition, we did not examine the possible direct effects of these diuretics on osteoclastic systems. Therefore, the possibility remains that thiazide or furosemide may influence other cytokines (not IL-6/IL-11) involved in the regulation of bone remodeling, or have direct effects on osteoclastic systems. Another potential limitation of our study is that we used second-passage cells and we cannot exclude the possibility that second-passage cells may have lost the ability to respond to the diuretics over the first passage. However, we have confirmed in previous studies that these second-passage cells maintain the characteristics of osteoblastic cells,[19,20,23] and we also reconfirmed in an adjunctive experiment that the hBMSC response to the diuretics was not different in the first-passage cells.

In summary, our results suggest that HCTZ and furosemide have no significant direct effect on the proliferation, alkaline phosphatase activity, and osteocalcin and IL-6/IL-11 production of hBMSCs, and the effects of these diuretics on bone mass may be due to an indirect action on calcium metabolism.

## ACKNOWLEDGMENT

The authors thank J.S. Hong, Asan Institute for Life Sciences, for technical assistance.

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