THE EFFECT OF A SYNTHETIC ANALOGUE OF PYROPHOSPHATE ON CALCIUM, MAGNESIUM AND PHOSPHORUS HOMEOSTASIS IN SHEEP

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Summary

Three female sheep were daily administered a pyrophosphate analogue, disodium 1-hydroxyethylidene-1, 1-bisphosphonate (HEBP) at the level of 4 mg/kg body weight. HEBP largely suppressed bone resorption, which was indicated by the reduction in plasma free hydroxyproline concentration and in calcium mobilization rate during the intravenous infusion of disodium ethylenediaminetetraacetate (EDTA). Contrary to the suppression of bone resorption, plasma total-calcium, magnesium and phosphorus concentrations were not changed by HEBP administration. These results suggest that bone mineral crystals play a meaningless role on calcium, magnesium and phosphorus homeostasis in ruminants if they are fed adequate amounts of these minerals. Plasma magnesium and phosphorus concentrations were not significantly changed after feeding. However, plasma total-calcium was decreased after feeding in both periods and the reduction seemed to be remarkable in the HEBP-treated period. Infusion of EDTA more remarkably reduced plasma ionized calcium concentration in the HEBP-treated than in the untreated period and the recovery of ionized calcium was retarded by HEBP administration. These results suggest that calcium release from bone is necessary for maintenance of plasma calcium when animals rapidly lose calcium.

(**Key Words**: Bisphosphonate, Calcium, Phosphorus, Magnesium, Bone, Sheep)

Introduction

Calcium (Ca), magnesium (Mg) and phosphorus (P) are important components of the hard tissues. Approximately, 99% of Ca, 70% of Mg and 80% of P in the whole body of adult ruminants locate in bones and teeth. It is recognized that Ca and P are constantly deposited and removed in the skeletal tissue, which plays important roles on Ca and P metabolism in mammals including ruminants (Georgievskii, 1982). Bone Mg is also exchangeable to Mg in extracellular fluid and is postulated to contribute, to some extent, to Mg homeostasis in ruminants (Simesen et al., 1964; House and Campen, 1971). Almost all of these results, on the other hand, have been obtained by kinetics studies and there are few evidences that directly show the importance of bony tissue to keep Ca, Mg and P homeostasis in ruminants.

It is suggested that mineral exchange between bone mineral crystals and extracellular fluid is regulated by pyrophosphate, i. e., pyrophosphate is adsorbed on the surface of bone mineral crystals to interfere with the precipitation and dissolution of minerals, and enzymatic degradation of pyrophosphate is involved in mineral movement (Fleisch et al., 1966a; Fleisch et al., 1966b). Although pyrophosphate administration, itself, dose not affect bone metabolism *in vivo* because of its rapid hydrolysis, some pyrophosphate analogues, which are resistant to the hydrolysis, have been developed (Fleisch, 1985). These analogues are now known to be highly active in the skeletal tissue of laboratory animals and have little toxicity.

The objectives of this study is to clarify the importance of bone in Ca, Mg and P homeostasis by measuring these mineral concentrations in plasma during the suppression of bone metabolism by the administration of a pyrophosphate analogue.

Materials and Methods

Three female sheep, aged 1 year and weighing about 28 kg, were used. Each sheep was housed in a separate pen and fed the diet shown in table 1, at a level of 1.1% of body weight at 12-hr

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TABLE 1. DIET COMPOSITION

Ingredient/Composition	Percentage
Ingredients:	
Timothy hay	60.0
Wheat bran	20.0
Barley grain	18.5
Sodium chloride	1.0
Calcium carbonate	0.5
Composition:	
Calcium	0.51
Magnesium	0.29
Phosphorus	0.44

intervals. Water was available at all time.

A pyrophosphate analogue, disodium 1-hydroxyethylidene-1, 1-bisphosphonate (HEBP; Procter and Gamble Co, Norwich, New York, U.S.A.) was dissolved in sterilized saline and adjusted pH to 7 by hydrochloric acid. The HEBP solution was intramuscularly injected at 4 mg/kg body weight for 41 days when animals were given morning feed.

After a 5-day preliminary period, following 3 experiments were conducted in this study; (1) blood were collected from the jugular vein before the administration of HEBP and at 3 day intervals for 21 days during the administration at 4 hr after morning feeding. Plasma total-Ca, Mg, P and free hydroxyproline (Hyp) concentrations were measured in these blood samples. (2) two days before and 23 days after the initiation of HEBP administration, sheep were sampled blood just before, and 2, 4, 6 and 8 hr after feeding. Plasma total-Ca, Mg and P concentrations were measured in these blood samples. (3) twenty five days before and 38 days after the initiation of HEBP administration, every sheep was fitted with bilateral siliconized jugular catheters. Two days after the catheterization, animals were infused disodium ethylenediaminetetraacetate (EDTA) through a catheter at 4.38 μmol/kg body weight/min for 60 min by a peristaltic pump. The solution was prepared to contain 2% EDTA in saline and was adjusted pH to 7.4 by hydrochloric acid. The solution was sterilized in an autoclave (121℃, 20 min) 1 hr before the infusion. Blood samples were taken through the other catheter prior to, and 15, 30, 45, 60, 75, 90, 120, 150, 180 and 240 min after the initiation of EDTA infusion. And plasma total-Ca and ionized Ca concentration were measured. The rate of Ca mobilization was calculated according to the method of Contreras et al. (1982) modified by Terashima et al. (1988).

Plasma total-Ca and Mg concentrations were determined by an atomic absorption spectrophotometry. Ionized Ca concentration was measured by an ionic meter (Sera-250, Horiba Ltd. Japan) and phosphorus concentration was analyzed by the method of Fiske and Subbarow (1925). Plasma free Hyp was measured by the method of Bergman and Loxley (1961).

The statistical significance were calculated by a paired t-test.

Results

Figure 1 shows changes of total Ca, Mg, P and free Hyp concentrations in plasma after the initiation of HEBP administration with time. Plasma Hyp concentration began to decrease 3 days after the initiation of HEBP administration and reached to the approximately half level of the untreated period 6 days after the initiation of HEBP administration. Then the level of plasma

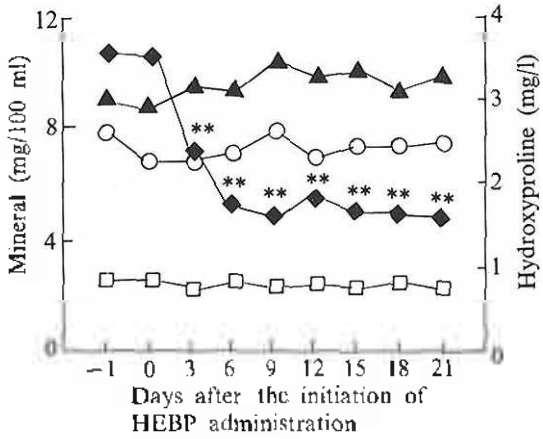

Figure 1. The effect of disodium 1-hydroxyethylidene-1, 1-bisphosphonate administration on plasma calcium (▲), magnesium (□), phosphorus (○) and free hydroxyproline (◆) concentrations in sheep.
** Significantly ($p < 0.01$) different from the value before the administration (−1 day).

Hyp was not changed till the end of the experiment. Contrary to the changes in plasma Hyp, plasma total-Ca, Mg and P concentrations were not changed by HEBP administration.

Figure 2 shows the changes of plasma total and ionized Ca concentrations by the intravenous infusion of EDTA in the untreated and the HEBP treated periods. EDTA infusion decreased plasma ionized Ca level of which recovery commenced at 30 min after the infusion in both periods. The reduction in ionized Ca concentration, however, was more remarkable in the HEBP-treated period than in the untreated period during EDTA infusion and the recovery of ionized Ca was retarded by HEBP administration after EDTA infusion. Consequently, plasma ionized Ca concentration perfectly recovered in sheep which were not administered HEBP but was approximately 40% less in the HEBP-treated animals than in the untreated ones 240 min after the initiation of EDTA infusion.

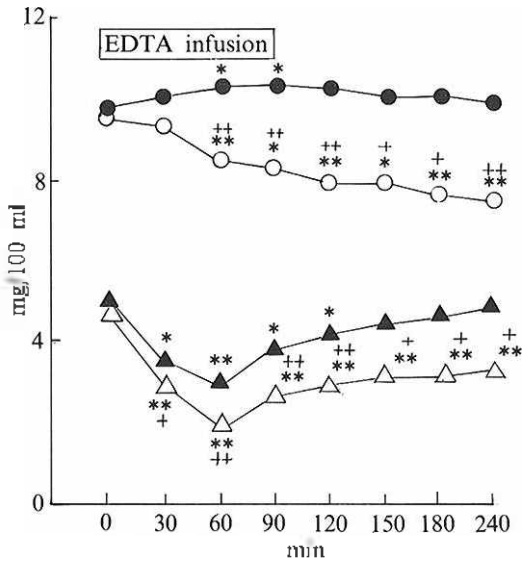

Figure 2. Changes in plasma total and ionized calcium concentrations during the intravenous infusion of disodium ethylenediaminetetraacetate (EDTA) in periods when sheep were or were not administered disodium 1-hydroxyethylidene-1, 1-bisphosphonate (HEBP).
○ : total calcium in the HEBP-treated period.
● : total calcium in the untreated period.
△ : ionized calcium in the HEBP-treated period.
▲ : ionized calcium in the untreated period.
Significantly (* $p < 0.05$, ** $p < 0.01$) different from the value before EDTA infusion. Significantly (+ $p < 0.05$, ++ $p < 0.01$) different from the value of the untreated period.

Plasma total-Ca concentration was slightly increased by EDTA infusion in the untreated period but was gradually decreased in the HEBP-treated period till the end of the experiment. Ca mobilization rates, which were calculated from the changes in plasma total and ionized Ca levels, were 228 ± 18 and 176 ± 11 (mean ± SE) mg/hr in the untreated and the HEBP-treated periods, respectively. There was a significant ($p < 0.01$) difference between these values.

Table 2 shows changes in plasma total-Ca, Mg and P concentrations after feeding in the untreated and the HEBP-treated periods. There were no significant changes in plasma Mg and P concentrations after feeding in both periods and HEBP administration did not affect plasma Mg and P concentrations after feeding. On the other hand, plasma Ca concentration was significantly ($p < 0.05$) decreased in the untreated period 4 hr after feeding and in the HEBP-treated period 2 and 4 hr after feeding and the reduction in plasma Ca seemed to be remarkable in the HEBP-treated period.

Discussion

Administration of HEBP strikingly decreased plasma free Hyp concentration which is thought to be an index of bone resorption (Dull and Hennemann, 1963). In addition, Ca mobilization was significantly suppressed by HEBP during EDTA infusion. These results indicate that HEBP largely interferes with bone resorption in sheep. The suppressive effect of HEBP on bone resorption was also found in various mammals (Gasser et al., 1972; Reynolds et al., 1972; Guncaga et al., 1974).

Administration of HEBP lowered plasma Hyp concentrations to more than 50% of the value in the untreated period. The reduction of Ca mobilization rate, however, was not so remarkable as the decrease in plasma Hyp. When EDTA

TABLE 2. THE EFFECT OF HEBP[a] ADMINISTRATION ON PLASMA MINERAL CONCENTRATIONS AFTER FEEDING IN SHEEP

	Before feeding	Hours after feeding			
		2	4	8	12
			Calcium (mg/100 ml)		
Untreated	8.87 ± 0.33	8.32 ± 0.49	8.35 ± 0.27*	8.58 ± 0.48	8.20 ± 0.42
Administered	8.80 ± 0.45	8.15 ± 0.25*	8.03 ± 0.35*	8.23 ± 0.30	8.62 ± 0.37
			Magnesium (mg/100 ml)		
Untreated	2.07 ± 0.22	2.06 ± 0.20	2.05 ± 0.16	2.08 ± 0.18	2.04 ± 0.14
Administered	2.30 ± 0.12	2.10 ± 0.08	2.20 ± 0.07	2.29 ± 0.07	2.32 ± 0.18
			Phosphorus (mg/100 ml)		
Untreated	7.45 ± 0.32	7.68 ± 0.64	7.38 ± 0.51	6.80 ± 0.49	7.01 ± 0.40
Administered	6.99 ± 0.60	6.98 ± 0.27	6.56 ± 0.34	6.78 ± 0.59	6.72 ± 0.39

Values are means ± SE (n = 3).
* Significantly ($p < 0.05$) different from the value before feeding.
[a] Disodium 1-hydroxyethylidene-1, 1-bisphosphonate.

is infused, Ca is possibly mobilized from not only bone but also some soft tissues. Furthermore, Ca can be released from the skeletal tissue without bone resorption. It is postulated that there is an easily exchangeable Ca pool between bone matrix, where minerals are precipitated as crystals, and bone cells including osteoblasts and osteocytes. The Ca pool is named bone fluid which is thought to be owing to a rapid Ca movement into general extracellular fluid (Talmage, 1970). Calcium in bone fluid possibly enters general extracellular fluid when plasma ionized Ca level is decreased by EDTA infusion. On the other hand, plasma free Hyp is suggested to be increased by collagen degradation in bone matrix (Dull and Hennemann, 1963) which is involved in Ca release from bone crystal. It seems reasonable that HEBP administration more remarkably decreases plasma Hyp than Ca mobilization rate measured by EDTA infusion.

Plasma total-Ca, Mg and P concentrations were not changed by HEBP administration though bone resorption was obviously suppressed. These results suggest that sheep can keep homeostasis of these minerals notwithstanding the striking suppression of bone resorption if they are fed adequate amounts of minerals. The suppression of bone resorption is thought to be compensated for these mineral metabolisms in the alimentary tract and the bone fluid, and, to the less extent, in the kidney. In addition, pyrophosphate analogues are reported to suppress not only bone resorption but also bone formation (Gasser et al., 1972). The suppression of bone anabolism may also affect maintenance of mineral homeostasis during HEBP administration.

Contrary to changeless in plasma Ca concentration during HEBP administration, HEBP promotes the reduction of ionized Ca level during EDTA infusion. These results indicate that Ca release from bone mineral crystals is necessary for maintenance of plasma Ca when Ca is rapidly lost. Furthermore it may not be impossible that a mineral homeostasis is disturbed by HEBP administration when sheep suffer a mineral deficiency. In fact, Mg deficiency induced severer hypomagnesemia in HEBP-treated sheep than in intact ones (Matsui et al., 1991). There are contradictory reports about the effect of HEBP administration on plasma Ca and P concentrations. Talmage et al. (1974) reported that HEBP administration at 40 mg/kg body weight/day did not affect serum Ca and P concentration in rats. On the other hand, Rosenblum (1974) described that dose of 2.5 mg/kg body weight/day HEBP did not affect serum Ca concentration but increased serum P in rabbits. The higher dose of HEBP administration was reported to increase Ca balance and plasma Ca in rats (Gasser et al., 1972). It is not clear why the contradiction occurs but physiological conditions of used animals, doses of HEBP and nutritional status of minerals might affect the response to HEBP administration.

In monogastric animals, it has been thought that the influx of Ca from the gut into extra-

cellular fluid, i.e., Ca absorption, is not constant and it may not be sufficient to maintain plasma Ca concentration during a period between meals. The gradual reduction in Ca absorption would stimulate bone resorption through the increase in the secretion of parathyroid hormone (Shaafsma, 1988). Furthermore it is suggested that postprandial stimulation of calcitonin secretion inhibits bone resorption and prevents hypercalcemia induced by the increase in Ca absorption after feeding (Munson, 1976).

The apparent absorption of Ca mainly occurs in the forestomach of ruminants (Grace et al., 1974). It is possible to consider that the rate of Ca absorption is not constant and that bone plays an important role on maintenance of Ca homeostasis which is apt to be disturbed by feeding in ruminants as same as in monogastric animals. In this study, plasma Ca level was decreased after feeding in the untreated period. The results may support that there is a mechanism to prevent hypercalcemia induced by feeding in ruminants as same as in monogastric animals. On the other hand, plasma Ca tended to be remarkably decreased in the HEBP-treated period, which indicated that the postprandial reduction in plasma Ca is not related to the suppression of bone resorption. It is, furthermore, suggested that the reduction in plasma Ca is not owing to the increasing calcitonin secretion in sheep because the hypocalcemic action of calcitonin is reported to be diminished by HEBP administration in rats (Talmage et al., 1974).

Milhaud et al. (1972) reported that plasma Ca tended to be decreased during the feeding period in intact rats but was increased in thyroidectomized ones. It was shown, however, that the decrease in plasma Ca during feeding was not reversed by thyroidectomy (Talmage et al., 1975). It is proposed from the latter report and the present study that a factor or factors other than calcitonin may induce the postprandial reduction in plasma Ca concentration without changing bone metabolism. Further study is needed to clarify the mechanism inducing the postprandial fall in plasma Ca.

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Literature Cited

Bergman, J. and R. Loxley. 1963. Two improved and simplified methods for the spectrophotometric determination of hydroxyproline. Anal. Chem. 35 :1961-1965.

Contreras, P. A., R. Manston and B. F. Sansom. 1982. Calcium mobilisation in hypomagnesaemic cattle. Res. Vet. Sci. 33:10-16.

Dull, T. A. and P. H. Hennemann. 1963. Urinary hydroxyproline as an index of collagen turnover in bone. N. Engl. J. Med. 268:132-134.

Fiske, C. M. and Y. Subbarow. 1925. The colorimetric determination of phosphorus. J. Biol. Chem. 66: 375-382.

Fleisch, H., J. Maerki and R. G. G. Russel. 1966a. Effect of pyrophosphate on dissolution of hydroxyapatite and its possible importance in calcium homeostasis. Proc. Soc. Exp. Biol. Med. 122:317 -320.

Fleisch, H., R. G. G. Russel and F. Straumann. 1966b. Effect of pyrophosphate on hydroxyapatite and its implications in calcium homeostasis. Nature (London) 212:901-903.

Fleisch, H. 1985. Chemistry and mechanisms of action of bisphosphonates. In: S. Garattini (ed.). Bone resorption metastasis, and diphosphonates. pp. 33 -40. Raven Press, New York.

Gasser, A. B., D. B. Morgan, H. A. Fleisch and L. J. Richelle. 1972. The influence of two diphosphonates on calcium metabolism in the rat. Clin. Sci. 43:31-45.

Georgievskii, V. I. 1982. The physiological role of macroelements. In: V. I. Georgievskii, B. N. Annenkov and V. T. Samokhin (eds.). Mineral nutrition of animals. pp. 91-170, Butterworths, London.

Grace, N. D., M. J. Ulyatt and J. C. Macrae. 1974. Quantitative digestion of fresh herbage by sheep. 3. The movement of Mg, Ca, P, K and Na in the digestive tract. J. Agric. Sci. Camb. 82:321-330.

Guncaga, J., Th. Lauffenburger, C. Lentner, M. A. Dambacher, H. G. Hass, H. Fleisch and A. J. Olah. 1974. Diphosphonate treatment of Paget's disease of bone. A correlated metabolic, calcium kinetic and morphometric study. Horm. Metab. Res. 6:62-69.

House, W. A. and D. V. Campen. 1971. Magnesium metabolism of sheep fed different levels of potassium and citric acid. J. Nutr. 101:1483-1492.

Matsui, T., T. Kawabata, T. Fujihara and T. Harumoto. 1991. The relationship between magnesium homeostasis and bone metabolism in sheep. In: M. Wan Zahari, Z. Ahmad Tajuddin, N. Abdullah and H. K. Wong (eds.). Proceedings of the third international symposium on the nutrition of herbivores. pp. 2. The Malaysian Society of Animal

Production, Serdang, Malaysia.

Milhaud, G., A. M. Perault-Staub and J. F Staub. 1972. Diurnal variation of plasma calcium and calcitonin function in the rat. J. Physiol. 222: 559- 567.

Munson, P. L. 1976. Physiology and pharmacology of thyrocalcitonin. In : G D. Aurbach (ed.). Handbook of physiology, section 7. vol. 7 pp 443-464 Waverly Press, Baltimore

Reynolds, J. J., C. Minkin, D. B. Morgan, D. Spycher and H. Fleisch. 1972. The effect of two diphosphonates on the resorption of mouse calvaria *in vitro* Calcif. Tissue Res. 10:302-313.

Rosenblum, I. Y. 1974. The effects of disodium ethan-1-hydroxy-1, 1-diphosphonate on bone and serum chemistry in rabbits Calcif. Tissue Res. 16:145-152.

Shaafsma, G. 1988. Calcium in extracellular fluid : homeostasis In : B E. C. Nordin (ed.). Calcium in human biology. pp. 241-259. Springer-Verlag, Berlin.

Simesen, M. G., T. A. Rogers, T. Lunaas and J. R. Luick. 1964. Plasma-Mg 28 disappearance in the bovine. Proc. Symp. Radioisot. Anim. Nutr. Physiol. pp. 721-736. International Atomic Energy Agnecy, Vienna.

Talmage, R. V. 1970 Morphological and physiological considerations in a new concept of calcium transport in bone. Am. J. Anat. 129:467 476.

Talmage, R. V., J. J. B. Anderson and J. W. Kennedy 1974 Separation of the hypocalcemic and hypophosphatemic actions of calcitonin with disodium ethan-1-hydroxy-1, 1-diphosphonate. Endocr. 94 413-418.

Talmage, R. V., J. H. Roycroft and J. J. B. Anderson. 1975. Daily fluctuations in plasma calcium, phosphate and their radionuclide concentrations in the rats. Calcif. Tissue Res. 17:91-102

Terashima, Y., S. Matsunobu, T. Yanagisawa and H. Itoh. 1988. Calcium mobilization in hypomagnesemic wethers fed on a low magnesium and/or high potassium diet. Jpn. J. Zootech. Sci. 59:75-81.