Effectiveness of the Elcatonin Transdermal System for the Treatment of Osteoporosis and the Effect of the Combination of Elcatonin and Active Vitamin D₃ in Rat

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The efficacy of percutaneous elcatonin (EC), a hypocalcemic peptide, in the treatment of experimental osteoporosis in rats was evaluated *in vivo*. Additionally, the effect of the combined use of EC and active vitamin D₃ (1,25(OH)₂D₃) for the treatment was compared with those of three other groups: 1,25(OH)₂D₃ alone, estradiol plus 1,25(OH)₂D₃, and a placebo, and low calcium diet (low Ca). The EC transdermal system and the EC plus 1,25(OH)₂D₃ system, applied to the rat abdominal skin 6 times for 48 h, significantly increased the ash weight and calcium content of the tibia in the rats, compared with those of placebo group ($p < 0.05$). The EC systems also slightly lowered the alkaline phosphatase activity in plasma of the morbid rats, without a difference in the plasma calcium content. These EC systems were superior to the 1,25(OH)₂D₃ system and the estradiol plus 1,25(OH)₂D₃ system in improving osteoporotic parameters. Thus, the EC systems were concluded to be an efficient drug delivery system for Paget's disease and osteoporosis.

Keywords elcatonin; elcatonin plus active vitamin D₃; transdermal system; experimental osteoporosis; bone formation; rat

The best known pharmacologic effects of calcitonin are to lower plasma calcium and inorganic phosphate concentrations by means of action on the major target organs: bone and kidney. The hypocalcemic peptide hormone has been used for the treatment of Paget's disease as well as certain types of osteoporosis.¹⁾ Elcatonin (EC), synthetic [Asu^{1,7}]-eel calcitonin, also inhibits osteoclastic bone resorption stimulated by bone resorptive factors.²⁾ Recently, there has been evidence that EC action can stimulate osteoblastic bone formation in addition to inhibiting bone resorption.³⁾

The process of delivering drugs through the skin for the systemic treatment of disease states has been brought into sharp focus in recent years by the efforts of pharmaceutical firms to develop transdermal delivery devices. We have found that EC is efficiently absorbed through rat skin in the presence of an absorption enhancer and a protease inhibitor, and that it exerts a potent hypocalcemic effect over a prolonged period.⁴⁾ Consequently, we have suggested that the transdermal delivery of EC has the potential to be an efficient drug delivery system for the treatment of Paget's disease and osteoporosis. However, the efficacy of this transdermal system in the treatment of osteoporosis has not been clarified.

The present study was designed to evaluate the efficacy of the EC system as a therapy to treat experimental osteoporosis in rats. Additionally, the effect of the combination of EC and active vitamin D₃ was compared with that of the transdermal delivery of EC or with a system involving both estradiol and active vitamin D₃ with the intention of developing a successful system for clinical use.

Materials and Methods

Materials [Asu^{1,7}]-eel calcitonin (6400 U/mg, Asahi Kasei Co.), a synthetic analogue of eel calcitonin, was used throughout this study. 1,25-Dihydroxy vitamin D₃ (1,25(OH)₂D₃) and 17- β -estradiol (estradiol) were purchased from Duphar Co. and Nacalai Tesque Inc., respectively. Carbopol 934, a gel base, and *n*-octyl- β -D-thioglucoside (OTG) were obtained from Kishida Chemical Co. and Wako Pure Chemical Co.,

respectively. A low calcium diet (modified A/N-B; Ca, 0.003%) was purchased from Oriental Yeast Co. Female Wistar rats (Japan SLC Inc.), weighing 100–110 g and then 200–220 g, were used throughout this experiment.

Preparation of Gel Ointment and Transdermal Therapeutic System (TTS) The gel ointment was prepared by the same method as described in a previous paper.⁴⁾ Some gel ointments were prepared after the addition of 1,25(OH)₂D₃ or estradiol and 1,25(OH)₂D₃ dissolved in propylene glycol in the same way. Details of the gel ointment composition are listed in Table I. TTSs were also prepared using a corresponding gel ointment described in Table I by the previous method (0.5 g/4 cm²).⁴⁾

Animal Experimental Procedures 1. Development of Experimental Osteoporosis: Female rats were divided at random into 3 groups, each consisting of 4–24 rats, weighing 100–110 g. One group (A) was ovariectomized under pentobarbital anesthesia (50 mg/kg, i.p.). The second group (B) underwent a sham operation. After that, both groups had free access to a low calcium diet for 2 months. The third group (C) underwent the sham operation and then had free access to a normal diet (MF-diet, Oriental Yeast Co.) for 2 months.

2. Treatment of Experimental Osteoporosis with TTS: Osteoporotic rats, weighing 200–220 g, were divided at random into 4 groups, each consisting of 4–6 rats. The hair of the abdominal area was carefully removed with an electric razor. In all groups, the low calcium diet was changed to a normal diet (MF-diet) beginning at that time. EC-TTS was applied to one group (EC group) on the shaved abdominal skin for 48 h, and the application of TTS was repeated 5 times at 2- or 3-d intervals. The second group (EC-1, 25(OH)₂D₃ group) and the third group (Est-1, 25(OH)₂D₃ group) were treated respectively with a TTS containing EC and 1,25(OH)₂D₃, and a TTS with estradiol and 1,25(OH)₂D₃,

TABLE I. Composition of Elcatonin, 1,25(OH)₂D₃ and Estradiol Gel Ointments

	Gel ointment				
	EC	EC+V.D ₃ ^{b)}	V.D ₃ ^{b)}	Est ^{c)} +V.D ₃ ^{b)}	Placebo
Carbopol 934	2.0 g	2.0 g	2.0 g	2.0 g	2.0 g
Elcatonin	25 mg	25 mg	—	—	—
1,25(OH) ₂ D ₃	—	5 μ g	5 μ g	5 μ g	—
17- β -Estradiol	—	—	—	10 mg	—
Gentamicin sol.	1.0 ml	1.0 ml	1.0 ml	1.0 ml	1.0 ml
OTG ^{a)}	1.5 g	1.5 g	1.5 g	1.5 g	1.5 g
Taurocholate	1.0 g	1.0 g	1.0 g	1.0 g	1.0 g
Purified water ad.	100 g	100 g	100 g	100 g	100 g

a) *n*-Octyl- β -D-thioglucoside. b) 1,25-Dihydroxy vitamin D₃. c) 17- β -Estradiol.

in the same manner. The fourth group (placebo group) was similarly treated with a placebo TTS. For comparison, the rats (control group) fed with MF-diet for 2 months and the rats (low Ca group) fed with low calcium diet for 2 months were treated with placebo ointment for 48 h 6 times, with both groups of rats being fed on MF-diet and low calcium diet during the treatment, respectively.

Measurement of Tibia Weight Both tibias of each rat were cut off from the legs and the cartilage tissue was removed. The wet and dry (after drying at 105 °C for 3 d) weights were taken.

Determination of Calcium and Phosphorus The tibia was ashed by heating at 600 °C for 5 h and the ash obtained was dissolved in 6N HCl. The calcium and phosphorus contents in the HCl solution and plasma were determined by the orthocresolphthalein complexone method using the Calcium C-Test Wako Kit (Wako Pure Chemical Co.) and Ames method,⁵⁾ respectively.

Assay of Alkaline Phosphatase Activity Alkaline phosphatase activity was assayed by the method of Kind and King,⁶⁾ using the Alkaline Phosphatase K-Test Wako Kit (Wako Pure Chemical Co.).

Determination of 1,25(OH)₂D₃ 1,25(OH)₂D₃ in gel ointment was determined by the method of the Shimadzu application data digest (CA 190—098). Briefly, 3 ml of methanol was added to 1 g of gel ointment and vigorously shaken. After centrifugation, the supernatant was injected into a reversed-phase Inertsil octadecyl silica (ODS) column (4.6 × 150 mm, GL Sciences Inc.) using a Shimadzu liquid chromatograph (model LC-6A) equipped with a UV spectrophotometer (model SPD-6AV). The mobile phase was 85% methanol. Detection was at 265 nm.

Statistical Analysis The means of all data are presented with their standard deviation (mean ± S.D.). One-way analysis of variance (ANOVA) with Scheffé's multiple comparison procedure was used to compare between-group differences, and a *p* value of 0.05 or less was considered to be significant.

Results

Identification of Experimental Osteoporosis The weight of the tibia and the calcium and phosphorus contents of tibia in the rats (A) ovariectomized and maintained on the low calcium diet are shown in Table II, along with the data of those rats (B) which underwent the sham operation and were fed on the low calcium diet as well as the rats (C) with the sham operation and a normal diet (Ca, 0.48%). These results indicated that the wet and dry weights of the tibia and the ash weight of group A were extremely and significantly lowered by the experimental treatment compared with those of group C (*p* < 0.001). Consequently, the calcium and phosphorus contents of the tibia of group A were also dramatically decreased compared with those of group C (*p* < 0.001). On the other hand, alkaline phosphatase activity in the plasma of group A was much more enhanced by the treatment than that of group C (*p* < 0.001). These low values and a high value observed with experimental osteoporosis agreed well with the data of rat osteoporosis reported previously.⁷⁾ Thus, rats with osteoporosis have surely benefitted by the treatment (A). Interestingly, the sham operation and the feeding of a low

calcium diet also lowered these parameters, the same as those of group A, suggesting the development of osteoporosis.

Treatment of Experimental Osteoporosis with Various TTSs Rats with osteoporosis developed by means of experimental protocol were treated with various TTSs for 48 h 6 times. The results are shown in Fig. 1 and Table III. The EC group significantly enhanced the ash weight and calcium content of the tibia compared with those of the placebo and low Ca groups (*p* < 0.05). Additionally, the EC group showed a slight, but not significant, decrease in alkaline phosphatase activity in plasma in comparison with the placebo group. Although the recovery rates of the parameters of the EC group did not reach the control levels (control group), our data certainly demonstrated the efficacy of the EC TTS for the treatment of experimental osteoporosis.

It has been reported that active vitamin D₃, such as 1α-OH D₃ and 1,25(OH)₂D₃,^{8,9)} and estrogen,^{10,11)} improve calcium absorption in patients with osteoporosis. These observations led to our interest in the combined use of EC and active V.D₃ for improving the therapeutic effect of the EC transdermal system. The effect of the combined use of EC and 1,25(OH)₂D₃ to treat rats with osteoporosis is shown in Fig. 1 and Table III, together with the effect of both estradiol and 1,25(OH)₂D₃. The data of rats treated with 1,25(OH)₂D₃ alone or with the placebo ointment (control), and of rats fed continuously on a low calcium diet (low Ca), are also depicted in the table and figure. These results indicate that the combined use of EC and 1,25(OH)₂D₃ resulted in a significant increase in the ash weight and calcium content of the tibia compared with the placebo group (*p* < 0.05). They also indicated a slight, but not significant, increase in the wet and dry weights of the tibia and in a slight reduction of the alkaline phosphatase activity in plasma compared with those of placebo group. On the other hand, the treatment with 1,25(OH)₂D₃ alone and estradiol plus 1,25(OH)₂D₃ (Est + 1,25(OH)₂D₃) showed no significant improvement in the osteoporotic parameters compared with the rats treated with the placebo ointment. The parameters after application of EC + 1,25(OH)₂D₃ were only slightly, but not significantly, higher than those after EC, as shown in Fig. 1A and B. Thus, it is suggested from the results that the combined use of EC and 1,25(OH)₂D₃ might be slightly more effective for the treatment of osteoporosis compared with the use of EC alone, and that EC or EC plus 1,25(OH)₂D₃ were superior to either 1,25(OH)₂D₃ alone or estradiol plus 1,25(OH)₂D₃ in the improvement of the parameters.

TABLE II. Effect of Ovariectomy and Feeding on Low Calcium Diet on Tibia and Plasma Components

Group	Body weight (g)	Tibia (mg)			Ash content of tibia					Plasma		
		Wet weight	Dry weight	Ash weight	Ca (mg)	P (mg)	Ca/dry weight (%)	P/dry weight (%)	Ca/P	Ca (mg/dl)	P (mg/dl)	Alkaline phosphatase (KAU)
A	210.0 ± 7.1 ^{a)}	344.6 ± 14.8 ^{a)}	227.6 ± 17.3 ^{a)}	93.3 ± 7.2 ^{a)}	32.2 ± 3.2 ^{a)}	17.0 ± 1.6 ^{a)}	14.2 ± 1.0 ^{a)}	7.5 ± 0.4 ^{a)}	1.90 ± 0.07 ^{a)}	8.9 ± 0.4	5.7 ± 0.5	36.9 ± 3.8 ^{a)}
B	202.5 ± 5.6 ^{a)}	341.9 ± 6.1 ^{a)}	208.7 ± 3.8 ^{a)}	90.4 ± 5.9 ^{a)}	31.1 ± 2.7 ^{a)}	16.1 ± 1.5 ^{a)}	14.9 ± 1.1 ^{a)}	7.7 ± 0.6 ^{a)}	1.94 ± 0.02 ^{a)}	8.7 ± 0.3	5.2 ± 0.7	35.2 ± 2.5 ^{a)}
C	207.5 ± 9.0	443.3 ± 7.3	333.0 ± 13.7	203.6 ± 8.7	74.7 ± 2.6	35.5 ± 1.6	22.4 ± 0.6	10.7 ± 0.4	2.11 ± 0.03	8.6 ± 0.4	5.5 ± 0.5	18.6 ± 1.5

A, ovariectomy + low Ca diet; B, sham operation + low Ca diet; C, sham operation + normal diet. a) *p* < 0.001 compared with group C. Rats were fed on each diet for 2 months after the operation.

Fig. 1. Effect of Elcatonin, 1,25 (OH)₂D₃ (V.D₃), Estradiol and Their Combined Use on Tibia Weight and Its Components

A: Ash weight: a) $p < 0.05$ in EC vs. placebo, b) $p < 0.01$ in EC + V.D₃ vs. placebo. Wet weight, tibia weight and ash weight: $p < 0.05$ in all groups vs. control or low Ca. □, wet weight; ▨, dry weight; ▩, ash weight. B: Calcium: a) $p < 0.05$ in EC vs. placebo or V.D₃. b) $p < 0.05$ in EC + V.D₃ vs. placebo, V.D₃ or Est + V.D₃. Phosphorus: c) $p < 0.05$ in EC + V.D₃ vs. V.D₃. Calcium and phosphorus: $p < 0.01$ in all groups vs. control or low Ca. □, calcium; ▨, phosphorus. C: Calcium/dry tibia: a) $p < 0.05$ in EC vs. V.D₃ or Est + V.D₃. Phosphorus/dry tibia: b) $p < 0.05$ in EC vs. V.D₃. Calcium/dry tibia: $p < 0.01$ in all groups vs. control or low Ca. Phosphorus/dry tibia: $p < 0.01$ in all groups vs. control and in EC or EC + V.D₃ vs. low Ca. □, Ca/dry tibia; ▨, P/dry tibia.

Discussion

Many investigations have concluded that a substantial proportion of patients with postmenopausal osteoporosis have impaired intestinal calcium absorption.^{9,12,13} Androgen therapy is effective in the treatment of that osteoporosis which complicates androgen deficiency.¹⁴ Calcitonin is also effective in diminishing hypercalcemia and decreasing the concentration of phosphate in the plasma of patients with hyperparathyroidism and osteolytic bone

TABLE III. Effect of Elcatonin, 1,25(OH)₂D₃ (V.D₃) and Estradiol or Their Combined Use on Plasma Components

Systems	Component		
	Ca (mg/dl)	P (mg/dl)	Alkaline phosphatase (KAU)
EC	8.83 ± 0.20	5.32 ± 0.35	23.69 ± 1.16 ^{a)}
EC + V.D ₃	8.80 ± 0.27	5.22 ± 0.36	22.21 ± 4.01 ^{a)}
V.D ₃	8.49 ± 0.53	5.08 ± 0.38	27.89 ± 3.56 ^{b)}
Est + V.D ₃	8.55 ± 0.50	5.22 ± 0.26	27.15 ± 3.17 ^{b)}
Placebo	8.74 ± 0.16	5.20 ± 0.35	26.57 ± 0.99
Control	8.94 ± 0.17	5.58 ± 0.55	18.83 ± 0.75
Low Ca	8.77 ± 0.34	5.32 ± 0.26	46.27 ± 2.28

a) Significantly different from control and low Ca, $p < 0.005$. b) Significantly different from control and low Ca, $p < 0.01$.

metastases. However, since calcitonins containing EC must be given by injection for therapy, calcitonin therapy is inconvenient for patients. A more simple or convenient administration may be favorable as a dosage route for EC and other calcitonins. To evaluate the efficiency of a percutaneous EC system which was previously developed by us, the EC system was applied to rats which had developed experimental osteoporosis. Additionally, the combined use of EC and 1,25(OH)₂D₃ was estimated in terms of the improvement of efficiency.

As a result, the EC system applied to rats with experimentally developed osteoporosis effectively increased the ash weight and calcium content of the tibia, and slightly, but not significantly, lowered the alkaline phosphatase activity in plasma, compared with a placebo group. This indicates that EC was absorbed through rat skin in the presence of absorption enhancers and that the EC system was effective for the therapeutic treatment of experimentally induced osteoporosis. Our previous study demonstrates that the bioavailability of the same EC system as that used in this study was 4.6% during a 24 h-application.⁴ In this study, the application time of the EC system was 48 h. Therefore, the absorption efficiency of EC would be increased more than 4.6%. To further improve the efficiency of the EC system, EC was used in combination with active V.D₃. The combined use only slightly, but not significantly, improved the increase in tibia weight and the calcium content of tibia compared with EC alone, but improved both factors more than 1,25(OH)₂D₃ alone or the estradiol plus 1,25(OH)₂D₃ systems. Therefore, it is suggested that the combined use of EC and 1,25(OH)₂D₃, in addition to the calcium supplement, may further enhance the efficacy of EC for the treatment of osteoporosis in man. This is interesting in comparison with the report that a combination of calcitonin with calcium supplements promises the effective treatment of osteoporosis and osteogenesis imperfecta.¹⁵ In addition, the stability of 1,25(OH)₂D₃ in gel ointments was measured after incubation at 37°C for 48 h. The recovery of 1,25(OH)₂D₃ after 48 h was 70.8 ± 13.6%. This indicates that the active V.D₃ would sufficiently retain its activity during the percutaneous experiments.

Interestingly, those rats which underwent the sham operation without ovario-steresis and were fed a low calcium diet developed the experimental osteoporosis, as

demonstrated by the various parameters (Table II). This suggests that long feeding on a low calcium diet will lead to the development of osteoporosis in rats, and probably in man.

Holst *et al.* demonstrate that the transdermal route is a viable means of delivering estradiol to systemic circulation.¹⁶⁾ Other workers clearly prove the percutaneous absorption of estradiol from a transdermal drug delivery system and the clinical efficacy of the system.¹⁷⁾ In this experiment, no remarkable improvement was obtained by the percutaneous administration of estradiol. Additionally, the combined use of estradiol and $1,25(\text{OH})_2\text{D}_3$ only partly improved the osteoporosis. The reason for the discrepancy between our results and the data reported previously¹⁶⁻¹⁸⁾ is unclear based on the results obtained. However, it may be partly related to the difference in the treatment term (Isaia *et al.* treated the women for one year).¹⁸⁾

In conclusion, the present results lead us to conclude that transdermal systems containing EC or EC plus $1,25(\text{OH})_2\text{D}_3$ significantly increased the ash weight and calcium content of tibia in rats with experimentally induced osteoporosis, compared with the placebo group. The efficacy was superior to that of either the $1,25(\text{OH})_2\text{D}_3$ or estradiol plus $1,25(\text{OH})_2\text{D}_3$ systems. It seems probable that these EC systems will offer an efficient drug delivery system for the treatment of Paget's disease and osteoporosis.

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