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Effect of Plasma-calcium-level-responsive Oestradiol Release from Apatitic Bone Cement on Bone Mineral Density in Ovariectomized Rats

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Abstract

The effects of plasma calcium levels on oestradiol release from apatite bone cement and on the bone mineral density of ovariectomized rats have been investigated.

Apatite cement was prepared from an equimolar mixture of tetracalcium phosphate, dicalcium phosphate dihydrate and 0·5% β-oestradiol bulk powder. After subcutaneous implantation of the cement, oestradiol release in diseased rats (ovariectomized rats on a low-calcium diet) was significantly higher than in normal rats. The drug levels of recovery-model rats (ovariectomized, but on a high-calcium diet) were significantly lower than those of the diseased rats. Calcium levels in diseased rats remained low during drug release but the plasma calcium levels of the recovery-model rats increased. The areas under the plasma calcium concentration–time curves (Ca-AUCs) for the recovery-model rats were higher than those for the diseased-model rats. The plasma oestradiol concentration AUCs and the Ca-AUCs were linearly related. The body weight of the recovery-model rats increased after five days, but that of the diseased-model rats did not. The bone mass of the recovery-model rats was greater after the experiment than before.

The relationship between the bone mineral density and Ca-AUC of the diseased rats suggested that bone mineral density increased with increasing Ca-AUC. The results suggest that the severity of osteoporosis in this animal model is reduced by implantation of the oestradiol-loaded apatite cement.

Because steroid hormones with oestrogenic activity such as oestradiol and oestrone are involved in the regulation of bone resorption and bone formation (Mclean et al 1968; Eriksen & Mosekilde 1990), bone remodelling becomes relatively inactive after menopause because of a decrease in the amount of the hormone and a subsequent decrease in the mineral density of the bone. Therefore, osteoporotic fractures are observed more commonly among post-menopausal women, because bone mechanical strength is closely related to bone mineral density (Lee et al 1981); these steroid hormones are, therefore, used to prevent osteoporosis (Baron et al 1987; Chapuy et al 1992). According to the National Nutrition Survey the recommended dietary intake of calcium (600 mg) has not been achieved in Japan over the past decade (Okano et al 1993), and calcium deficiency is another important factor in osteoporosis in Japan (Okano et al 1993). Okano et al (1993, 1994) reported that the bioavailability of calcium products was also closely related to bone mineral density in vitamin D-deficient rats, because plasma calcium concentrations and bone mineral density were lower in vitamin D-deficient rats.

We have previously designed a delivery system for several drugs (Otsuka et al 1995) using a biocompatible self-setting apatite cement (Brown & Chow 1986; Corltz et al 1995) which is transformed into hydroxyapatite in the body. We reported (Otsuka et al 1997a) that in-vitro drug release from apatite cement in simulated body fluid

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(Kokubo et al 1991) was dependent on the calcium level in the buffer. Because plasma calcium levels are low in calcium and vitamin D-deficient ovariectomized rats (Mclean et al 1968; Eriksen & Mosekilde 1990), we addressed this problem with our apatite cement-based drug-delivery system and demonstrated (Otsuka et al 1997b) plasma-calcium-level-responsive in-vivo oestradiol release from apatite cement in calcium- and vitamin D-deficient ovariectomized rats. However, to reverse the reduced bone mineral density associated with osteoporosis, relatively large doses of plasma calcium and anti-osteoporosis drug need to be delivered. In this study, therefore, we have studied the therapeutic effect on bone mineral density of self-regulated in-vivo oestradiol release from apatite cement.

Materials and Methods

Self-setting apatite cement

A 0·5% β-oestradiol-loaded apatite cement consisting of an equimolar mixture of tetracalcium phosphate ($Ca_4(PO_4)_2O$) and dicalcium phosphate dihydrate ($CaHPO_4.2H_2O$) was prepared as described previously (Otsuka et al 1997a,b). Calcium phosphate cement powders (0·50 g) were mixed with phosphoric acid (25 mM, 0·20 mL) for 1 min to form a paste, then the oestradiol bulk powders (2·5 mg) were mixed homogeneously with this paste. The final paste was poured into a 15-mm diameter plastic mould and stored at 37°C and 100% relative humidity for 24 h. The resulting hardened cement pellets were removed from the mould and embedded in silicone rubber, so that only one face of the pellet surface (1·77 cm^2) was exposed. The total weight of a cement pellet was 480 ± 5 mg.

Animal experiments

Animal care was conducted according to the Animal Care Committee guidelines of Kobe Pharmaceutical University. Diseased (i.e. ovariectomized rats on a low-calcium diet) or healthy rats, 150–170 g, were used. Female Wistar rats (SLC, Japan), five weeks old (approx.), 100 g average, were ovariectomized, divided into six groups, housed in multiples of four rats per cage, and fed a vitamin D- and Ca-deficient diet (Diet 11, Tables 1 and 2) (Suda et al 1970) or a normal diet for six weeks. Before the experiments the plasma calcium levels of the diseased and healthy rats averaged 5·1 and 10·4 mg/100 mL, respectively.

A drug-loaded cement device containing 2·5 mg oestradiol was implanted in the subcutaneous tissue on the backs of the diseased rats under anaesthesia induced by intraperitoneal administration of

Table 2. Composition of mineral mixture.

Ingredient	Amount (g/100 g)
KCl	57·5
NaCl	20·8
$MgSO_4$	17·8
$FeSO_4.7H_2O$	3·2
$CuSO_4.5H_2O$	$7{\cdot}8 \times 10^{-2}$
NaF	$11{\cdot}2 \times 10^{-2}$
$CoCl_2.6H_2O$	$3{\cdot}8 \times 10^{-3}$
KI	$8{\cdot}9 \times 10^{-3}$
$MnSO_4.H_2O$	$3{\cdot}9 \times 10^{-2}$
$ZnSO_4.7H_2O$	$4{\cdot}4 \times 10^{-1}$
$(NH_4)_6MoO_{24}.4H_2O$	$5{\cdot}0 \times 10^{-3}$

Table 1. Composition of diet.

Ingredient	Amount in normal diet (g/100 g)[a]	Amount in recovery diet (g/100 g)[a]	Amount in diseased diet (g/100 g)[a]
Glucose monohydrate	63·5	65·0	66·5
Vitamin-free casein	18·0	18·0	18·0
Cysteine	0·2	0·2	0·2
Choline chloride	10·0	10·0	10·0
Cottonseed oil	3·0	3·0	3·0
Vitamin D free vitamin mixture	0·1	0·1	0·1
Vitamin D3 (int. units)	250	0	0
Equimolar mixture of KH_2PO_4 and K_2HPO_4	0·20	0·2	0·2
Ca- and P-free mineral mixture	2·0	2·0	2·0
Calcium carbonate	3·0	1·5	0·0

[a]Except vitamin D3.

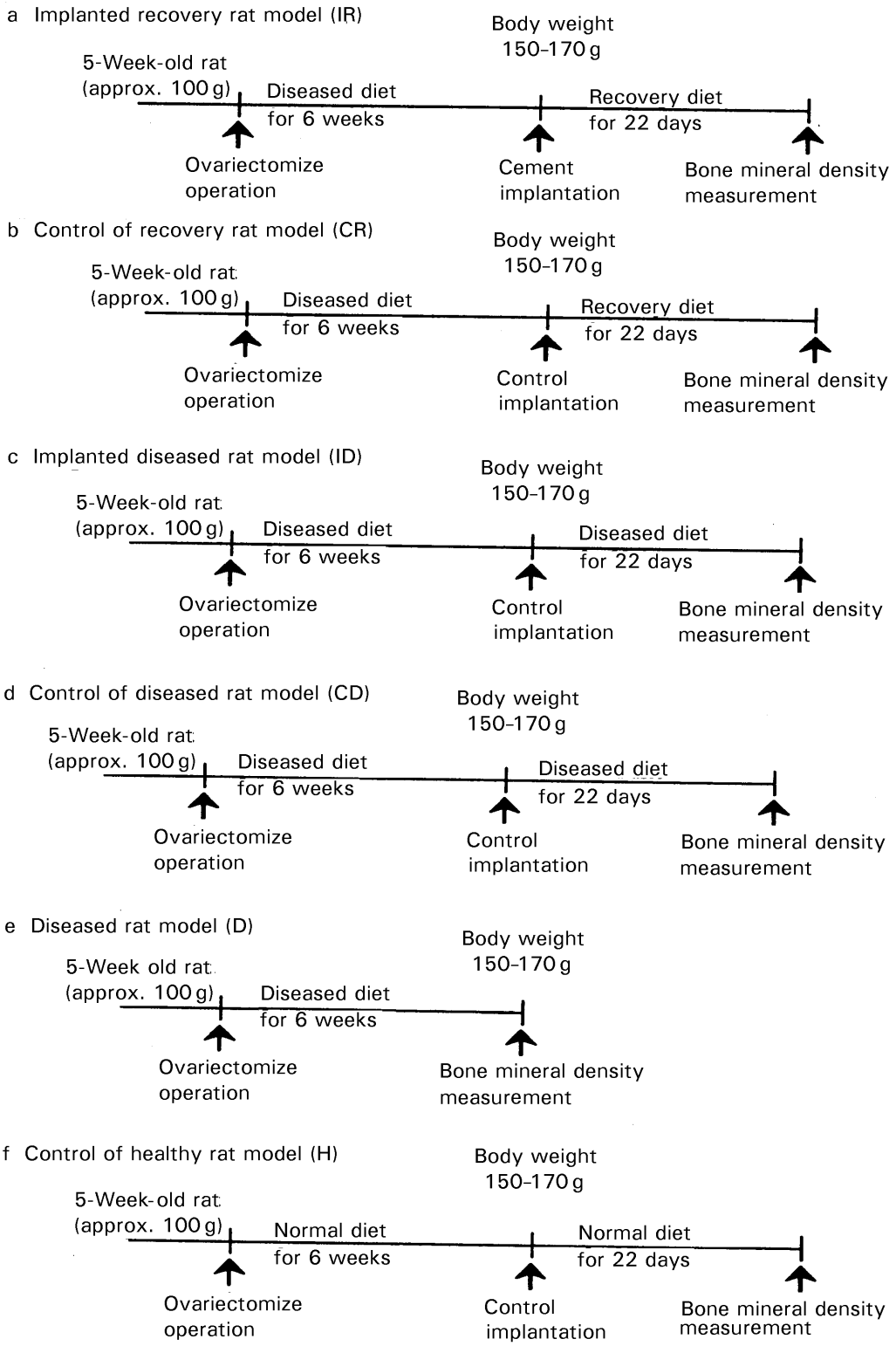

Figure 1. Protocol of animal experiments for determination of oestradiol release from drug-loaded apatite cements.

sodium pentobarbital (45 mg kg^{-1}). The diseased-model rats (groups ID and CD) were then fed Diet 11, and the recovery-model rats (groups IR and CR) were fed a normal diet containing 0·5% w/w calcium, as $CaCO_3$, for three weeks, during the drug-release test. Blood samples were collected after 12 h, and 1, 2, 4, 6, 8, 11, 14 and 22 days from the tail artery. The animal experimental schedules are shown in Figure 1. Four rats were used in each experiment.

Measurement of bone mineral density
The mineral density of the lumbar vertebrae of diseased and healthy rats was measured by bone mineral densitometry (Aroka model DCS-600R).

Radioimmunoassay of oestradiol
Plasma oestradiol was measured by means of a radioimmunoassay kit (Diagnostic Products, Los Angeles, CA). Measurements obtained by use of this kit were accurate for concentrations from 8 to 3600 pg mL^{-1}. Background levels of plasma oestradiol in control rats were $0{\cdot}0543 \pm 0{\cdot}016$ ng mL^{-1}.

Plasma calcium measurements
Plasma calcium concentrations were determined by the method of Kitano & Ueda (1971) which entails spectrophotometric measurement at 610 nm (UV 160A; Shimadzu, Kyoto, Japan) of the complex formed between methylxylenol blue and calcium ions.

Results and Discussion

Effect of plasma calcium level on oestradiol release from apatite cement in healthy and diseased rats
Figure 2 shows the plasma oestradiol concentration profiles of diseased and recovery-model rats after subcutaneous implantation of oestradiol-loaded apatite cement. Plasma oestradiol levels in the diseased-model rats (group ID) increased rapidly, and reached its maximum of $3{\cdot}3$ ng mL^{-1} after 12 h. Thereafter, the level decreased gradually but after 22 days was $0{\cdot}47$ ng mL^{-1} and remained significantly higher than that for the healthy group (group H), with release continuing after 22 days. In the recovery-model rats (group IR), the plasma oestradiol level increased rapidly, reaching a maximum concentration of $3{\cdot}1$ ng mL^{-1}. These levels, however, decreased gradually, and after 10 days were almost the same as those of group H, and after 22 days were $0{\cdot}32$ ng mL^{-1}. In contrast, the highest concentration in group H ($1{\cdot}45$ ng mL^{-1}) was much lower than those of groups ID and IR, and all levels were significantly lower than those of group ID. The drug levels at the later stage (7–22 days) for group IR were significantly lower than those for group ID, because the plasma calcium concentration for group IR was significantly higher than that for group ID, as shown below. This high plasma calcium concentration inhibited oestradiol release from the cement by precipitation of hydroxyapatite or by bone growth on the cement, or both, as reported previously (Otsuka et al 1997b).

Figure 3 shows the plasma calcium concentration profiles after subcutaneous implantation of oestradiol-loaded apatite cement in diseased and recovery rats. The plasma calcium level in healthy rats (group H) was (approx.) $10{\cdot}0 \pm 1{\cdot}2$ mg $(100\,mL)^{-1}$. In groups ID and CD, a level of approximately 5 mg/100 mL was maintained during drug release. In contrast, the plasma calcium level of groups IR and CR increased slightly, and reached (approx.)

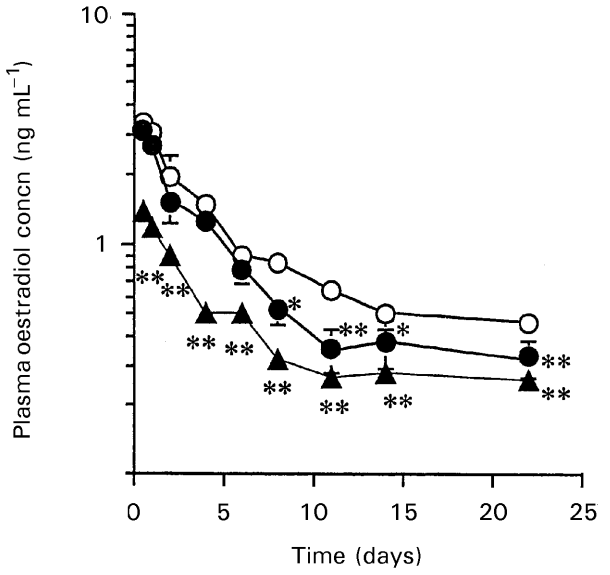

Figure 2. Plasma oestradiol levels of diseased and healthy rats after subcutaneous administration of oestradiol-loaded apatite cements: ID model (○), IR model (●), H model (▲). Each point and bar represent the mean and standard deviation. $*P < 0{\cdot}05$, $**P < 0{\cdot}01$ compared with ID model (Student's *t*-test, n = 4).

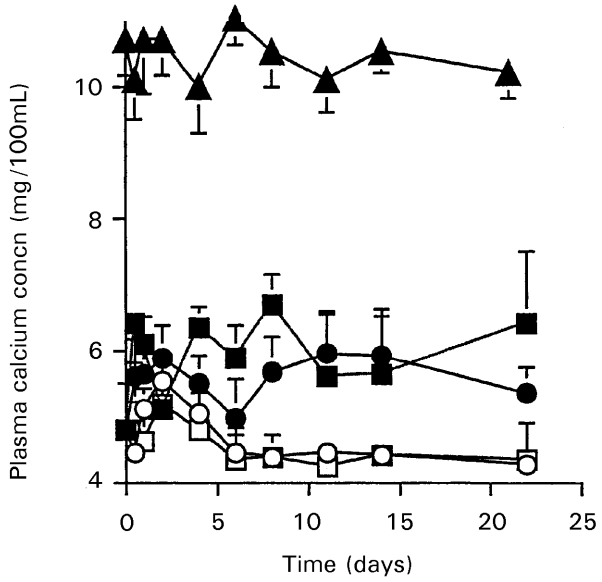

Figure 3. Plasma calcium levels of diseased and healthy rats after subcutaneous administration of oestradiol-loaded apatitic cements. ID model (○), IR model (●), CD model (□), CR model (■), H model (▲). Each point and bar represent the mean and standard deviation (n = 4).

Table 3. Effect of oestradiol-loaded cement on plasma calcium concentration.

Model	Food	Implant cement	Ca-AUC$_{0-22}$[a] (mg day/100 mL)
Control of diseased rat (CD)	Diseased diet	None	97·9 ± 12·9
Implanted diseased rat (ID)	Diseased diet	Cement	99·0 ± 17·0
Control of recovery rat (CR)	Recovery diet	None	134·4 ± 35·1
Implanted recovery rat (IR)	Recovery diet	Cement	122·4 ± 21·9
Control of healthy rat (H)	Normal diet	Cement	217·3 ± 19·2

[a]Area under the plot of plasma calcium concentration against time for 22 days ± s.d. (n = 4).

6 mg 100 mL after seven days. The area under the curve of plasma calcium concentration (Ca-AUC) for 22 days is summarized in Table 3. Ca-AUC for group IR was higher than for group ID, and those for groups IR and CR were higher than those for groups ID and CD, but the differences were not significant. It seems that it takes time to produce a therapeutic effect, such as an increase in plasma calcium level, by oestradiol release and calcium feeding, because the amount of calcium in the recovery diet was half that of the normal diet.

Figure 4 shows the linear relationship between Es-AUC (oestradiol-AUC) of oestradiol from apatite cement and Ca-AUC. Linear regression analysis revealed a significant ($P < 0.05$) relationship between Es-AUC and Ca-AUC; the correlation coefficient was 0·896. This result indicated that the Es-AUC of oestradiol-loaded cement was regulated by the total amount of plasma calcium during the drug-release test.

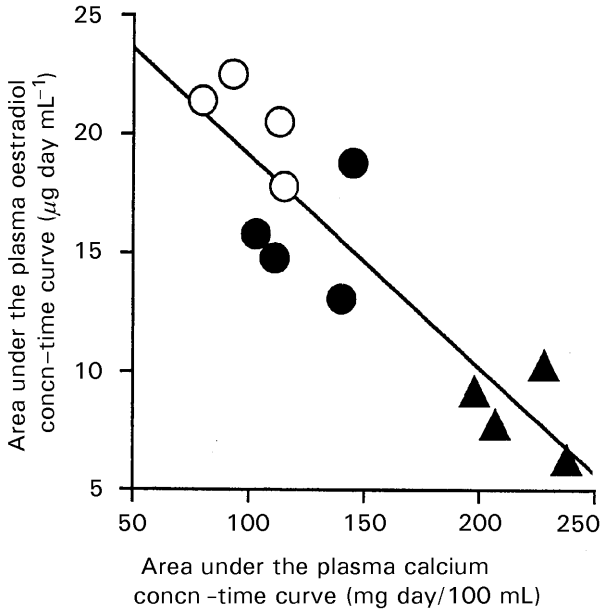

Figure 4. The relationship between the area under the oestradiol concentration–time curve from apatite cement and the area under the plasma calcium concentration–time curve; the equation of the line was $y = -0.090x + 28.080$ ($r = 0.896$). ID model (○), IR model (●), H model (▲). Each point and bar represent the mean and standard deviation (n = 4).

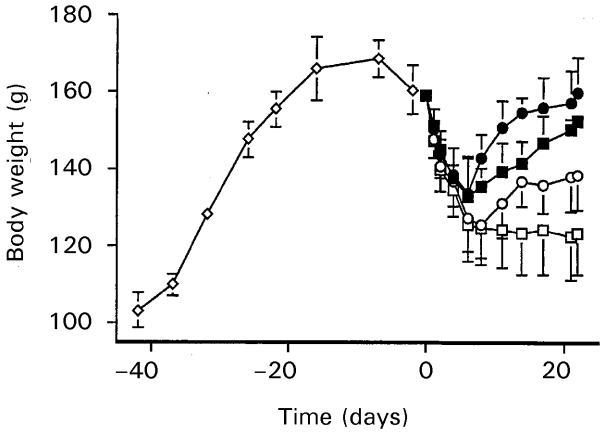

Figure 5. Weight change during oestradiol release in ovariectomized rats; implant operation is at time 0. ID model, implanted−Ca (○), IR model, implanted + Ca (●), CD model, control−Ca (□), CR model, control + Ca (■). Each point and bar represent the mean and standard deviation (n = 4 except n = 16 before implant (◇)).

Effect of oestradiol release from apatite cement on the bone mineral density of diseased rats

Figure 5 shows the body weight change of the rats during the apatite cement experiment. After the operation the body weight of ovariectomized rats increased until four weeks; after five weeks it decreased as a result of notarization effect. After the cement implant operation the weight decrease was accelerated by the physical damage of surgical operation. However, the body weight of groups IR and CR recovered after five days, and group ID gained weight after eight days, although group CD did not. The final body weights of the rats decreased in the order IR > CR > ID > CD. Because the increase in body weight was related to total bone mass, it is likely that body-weight differences reflected the therapeutic effect of the oestradiol or of calcium feeding.

Figure 6 shows the densitograms of bone mineral density in diseased and recovery-model rats after

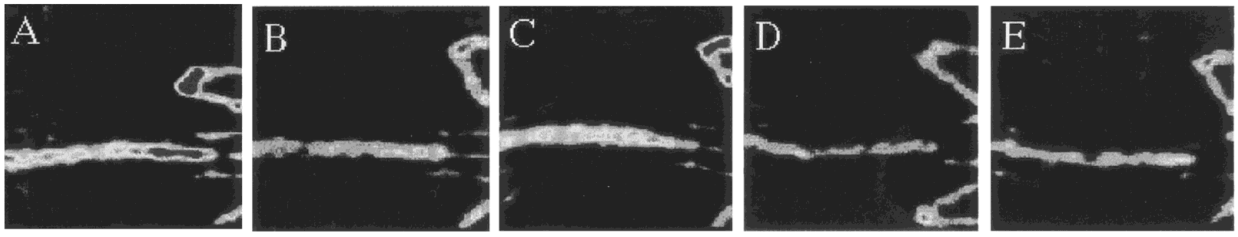

Figure 6. Densitograms of bone mineral in diseased rats after oestradiol release for 22 days. A, IR model, implanted + Ca; B, ID model, implanted−Ca; C, CR model, control + Ca; D, CD model, control−Ca; E, before implantation.

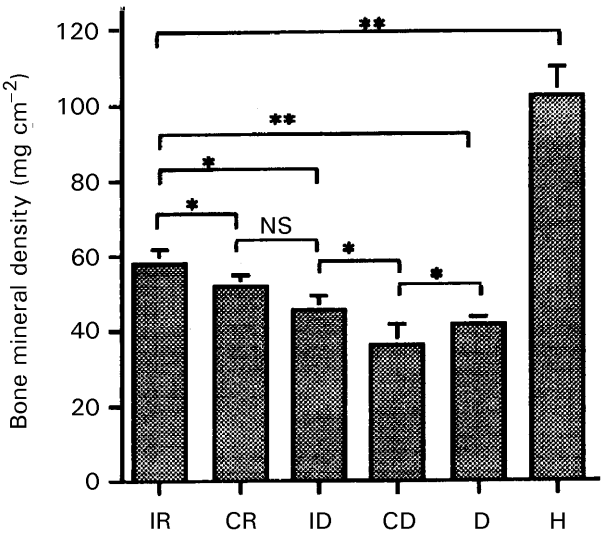

Figure 7. Bone mineral density of diseased rats after oestradiol release for 22 days. Each bar represents the mean and standard deviation. $*P < 0{\cdot}05$, $**P < 0{\cdot}01$ significantly different (Student's t-test, n = 4). D is the group CD (control−Ca) before cement implantation.

oestradiol release for 22 days. The results indicate that the bone mass of both diseased (ID) and recovery model (IR) rats was greater after the oestradiol release experiment than before implantation. The bone mass and bone mineral density differences reflected the therapeutic effect on the diseased rats of the oestradiol-loaded cement implantation or of calcium feeding, or both.

Figure 7 shows the bone mineral density of diseased and recovery-model rats after oestradiol release for 22 days. The bone mineral density for groups IR and CR was significantly higher than before the experiment, and significantly higher for group IR than for group CR, indicating that calcium feeding was an important factor and oestradiol loaded-cement implantation was effective in the therapeutic improvement of diseased-model rats. However, the bone mineral density of group ID was not significantly different from that before the experiment (group D), indicating that the oestradiol-loaded cement might not have a therapeutic effect if the plasma calcium concentration is insufficient. The bone mineral density of the diseased rats decreased in the order IR > CR > ID > CD, indicating that the increase in bone mineral density was a combination of the effects of oestradiol release from the cement and of plasma calcium concentration.

Conclusion

Because bone mass and density was regulated by the calcium supplement from the plasma and the activity of the bone cells in the rats, the increase of bone mineral density depended both on plasma oestradiol and on calcium concentration. The results suggest that plasma oestradiol levels are regulated by the characteristics of apatite cements, and the extent of osteoporosis is improved by implantation of oestradiol-loaded apatite cement with sufficient calcium supplement.

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