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Hormonal replacement therapy in an animal model with chronic renal failure and ovariectomy: Biochemical and densitometric study

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Background. In spite of estrogen replacement therapy being extensively used in clinical and experimental studies without renal impairment, there are no long-term studies concerning estrogen replacement in chronic renal failure.

Methods. In this experimental study, six groups of nephrectomized and ovariectomized animals were treated with different doses of 17β-estradiol, alone or in combination with calcitriol, to evaluate the effect of these treatments on bone metabolism.

Results. Biochemical results showed that estrogen alone did not have any effect neither on calcium nor on PTH serum levels. By contrast, in the groups treated with calcitriol, the levels of serum calcium were significantly higher, and the levels of iPTH were significantly lower than those observed in the control group. Animals receiving the combined treatment with estrogen and calcitriol showed the greater gain in uterus weight and a better bone mineral density at the lumbar site and the proximal and distal tibia sites.

Conclusion. The combination of estrogen and calcitriol is the most effective therapy to prevent bone mass loss in animals with chronic renal failure and estrogen deprivation.

Bone disease in chronic renal failure is a complex metabolic disorder. The alterations in bone metabolism vary depending on different factors, among which are age, sex, cause of renal failure, and, above all, the treatment received both in the predialysis and the dialysis phases [1]. Hypocalcemia, a decrease in 1,25-dihydroxy vitamin D syntheses, and the retention of phosphate are the most important factors in the development of secondary hyperparathyroidism [2, 3] and in the triggering of bone disturbances.

The deficiency of calcitriol plays an important role in the genesis of bone disease and explains its use in the management of hyperparathyroidism, which has been going on for nearly 30 years and which can be conceptually considered, at least partially, as a form of hormonal replacement therapy [4]. The administration of 1,25-dihydroxy vitamin D improves the intestinal calcium absorption, increases both serum calcium levels and urinary calcium excretion, and inhibits the secretion of PTH [5].

Apart from the three main regulators of bone metabolism, Ca, P, and calcitriol, there are others factors that can be implicated on bone metabolism. These factors are acidosis, aluminium, magnesium, local factors, and hormones such as estrogens.

Although there have been many clinical and experimental studies demonstrating that estrogen reduces bone loss and fractures in postmenopausal women [6, 7], little is known about the possible role that estrogen deficiency plays in the pathogenesis and progression of bone disease in chronic renal failure [8].

Taking into account the decrease of calcium absorption and the decrease of the estrogen levels that occur after menopause, it is likely that the combined therapy with estrogen and calcitriol has a synergic or additive effect on bone in uremic patients. For this reason, the aim of this study was to evaluate the effect of replacement treatment with different doses of 17β-estradiol (E_2), alone or combined with calcitriol on bone metabolism in an experimental model with nephrectomized and ovariectomized rats.

METHODS

Animals and diet

Six-month-old female Sprague-Dawley rats, with a mean body weight of 325 ± 32 g, were acclimatized under standard laboratory conditions at 24 ± 2°C and 50% to 60% humidity for one week. The rats were al-

Key words: estrogens, calcitriol, bone mass.

Table 1. Analysis of the different serum biochemical markers in the different treatment groups at the end of the study

	Reference normal group ($N = 5$)	CRF (without OVX) ($N = 4$)	CRF + OVX					
			Placebo ($N = 7$)	E2-15 ($N = 8$)	E2-45 ($N = 5$)	CT ($N = 7$)	E_2–15 + CT ($N = 6$)	E_2–45 + CT ($N = 7$)
Final body weight *g*	310 ± 26	321 ± 24	355 ± 34	313 ± 19[a]	333 ± 9[a]	350 ± 56	320 ± 12[a]	318 ± 13[a]
Urea *mg/dL*	31 ± 5	70 ± 11	68 ± 3	72 ± 14	65 ± 6	69 ± 6	71 ± 5	74 ± 15
Total proteins *g/L*	71.2 ± 1.4	68 ± 1	67 ± 3	70 ± 4	68 ± 5	69 ± 4	71 ± 6	74 ± 4[a]
Ca *mg/dL*	11 ± 0.44	12.0 ± 0.3	11.8 ± 0.3	11.7 ± 0.3	11.6 ± 0.1[a]	13.0 ± 1[a]	12.6 ± 0.3[a]	13.5 ± 0.8[a]
P *mg/dL*	4.8 ± 0.6	4.8 ± 0.6	5.7 ± 0.9	4.7 ± 0.6[a]	4.5 ± 0.8	5.7 ± 0.9[a]	5.3 ± 0.8	6.2 ± 0.6a
Alkaline phosphatase *UI/L*	244 ± 65	198 ± 42	245 ± 61	173 ± 52[a]	271 ± 84[a]	208 ± 65	228 ± 58	94 ± 19[a]
PTH *pg/mL*	14.8 ± 6.0	12.4 ± 2.1	18.7 ± 7.3	17.3 ± 9.0	12.1 ± 3.3	4.1 ± 2.7[a]	3.6 ± 1.2[a]	9.3 ± 5.6[a]
17β-E_2 *pg/mL*	45.2 ± 22.6	32.0 ± 13.0	12.2 ± 3.07[a]	23.6 ± 13.5[a]	35.9 ± 3.3[a]	12.1 ± 3.1[a]	18.9 ± 9.9	28.7 ± 6.7[a]

[a] $P < 0.05$

lowed free access to tap water and commercial standard rodent chow containing 0.6% calcium, 0.6% phosphorus, and 1500 kg of vitamin D_3 (Diet A04, Panlab SL, Barcelona, Spain).

Experimental design

Chronic renal failure (CRF) was surgically induced using the technique modified by Ormrod and Miller [9] (equivalent to 7/8 nephrectomy). Estrogen deprivation was obtained using bilateral ovariectomy (OVX) [10]. Both procedures were performed in the same surgical intervention using intraperitoneal ketamine hydrochloride (Ketolar) and medetomidine (Domtor) as anesthetics at dosages of 42 and 0.16 mg/kg, respectively. One week after the surgery, the group with CRF+OVX were divided into six treatment groups. Group 1 received the vehicle (corn oil) at a dose of 0.8 mL/kg body weight (BW)/day ($N = 7$) administered daily intraperitoneally for eight weeks. Group 2 received 17β-estradiol (E_2) (Sigma Chemical Co., St. Louis, MO, USA) dissolved in small amounts of ethanol with the volume adjusted with corn oil to give a concentration of 15 μg/kg/BW/day and administered daily intraperitoneally for eight weeks ($E_2 - 15$, $N = 8$). Group 3 received E_2 at a dose of 45 μg/kg/BW/day administered daily intraperitoneally for eight weeks ($E_2 - 45$, $N = 5$). Group 4 received calcitriol [1α,25$(OH)_2D_3$] (Sigma Chemical Co., St. Louis, MO, USA) dissolved in small amounts of ethanol with the volume adjusted with corn oil to give a concentration of 10 ng/kg/BW/day administered daily intraperitoneally for eight weeks ($N = 7$). Group 5 received $E_2 - 15$ in combination with calcitriol 10 ng/kg/BW, both administered daily intraperitoneally for eight weeks ($N = 6$). Group 6 received $E_2 - 45$ in combination with calcitriol 10 ng/kg/BW, both administered daily intraperitoneally for eight weeks ($N = 7$). The group with CRF ($N = 4$) without bilateral ovariectomy was used as the control group. In addition, a group of animals of the same age without any manipulation ($N = 5$) was used as the normal reference group. All rats were sacrificed by exsanguination after eight weeks, and their uteruses were removed and weighed for their use as markers of estrogen replacement.

Biochemical studies

Blood was obtained after sacrifice. The serum urea, calcium, phosphorus, and alkaline phosphatase, as well as the total proteins, were measured using a multi-channel autoanalyzer (Hitachi 717, Boehringer Mannheim, Berlin, Germany). The serum concentration of N-terminal PTH was measured using a chicken anti-PTH antibody (CK67.57) in an IRMA Rat PTH (Inmunotopics, San Juan Capistrano, CA, USA). Levels of 17β-estradiol were measured by radioimmunoassay (RIA) (Diagnostic Systems Laboratories, Inc., Webster, TX, USA).

Bone mineral measurements

Bone mineral density (BMD) was measured by dual-energy x-ray absorptiometry (DXA; Hologic QDR 100, Bedford, MA, USA), adapted to the measurement of BMD in small animals. These measurements were performed in vivo at the lumbar vertebra site, which included the first five lumbar vertebrae (using the last rib as reference), and ex vivo at the isolated right tibias in three segments: total, one-eight proximal, and seven-eight distal sites, according to Gómez Alonso et al [11].

Statistical analysis

Statistical analysis was performed using the Mann-Whitney test as a nonparametric test on SPSS 8.0 software for Windows (SPSS, Inc., Chicago, IL, USA). The results were expressed as mean ± standard deviation; differences were considered significant at a level of $P < 0.05$.

RESULTS

Biochemical parameters

The analysis of the different biochemical parameters is shown in Table 1. At the end of the experiment, all of the nephrectomized groups had similar serum urea levels (71 ± 10 mg/dL), which were significantly higher than those observed in the reference group of the same

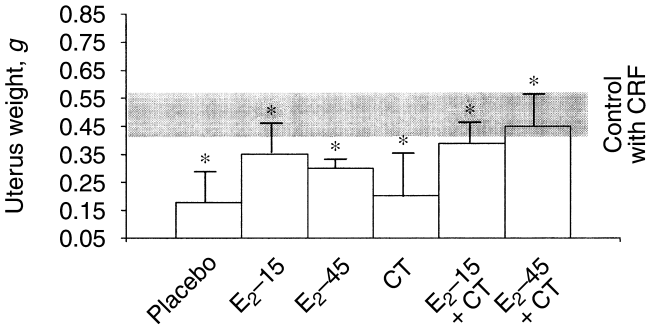

Fig. 1. Uterus weight in the different treatment groups. The gray bar shows the mean ± standard deviation in the CRF control group. Uterus weight in the reference normal group was 0.496 ± 0.161 g ($N = 5$). *$P < 0.05$

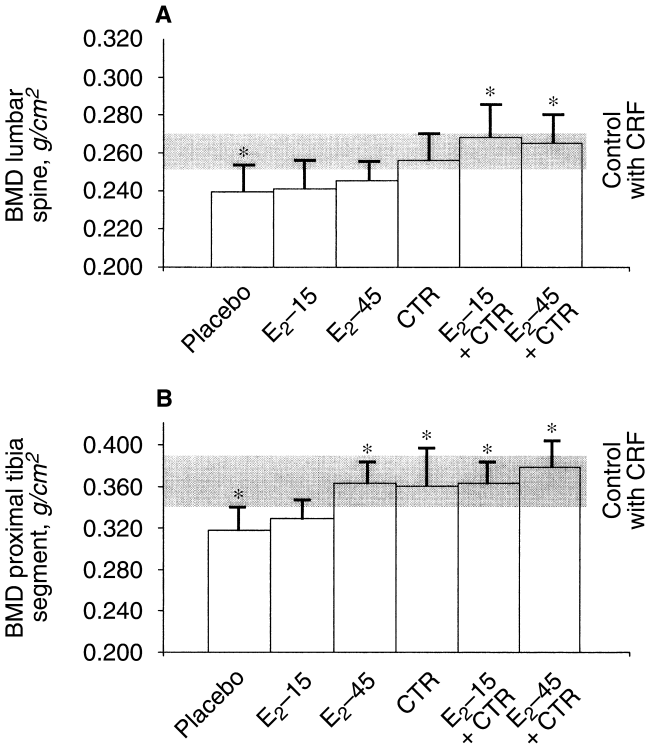

Fig. 2. BMD at the lumbar spine and the proximal tibia segment. (*A*) The gray bar shows the mean ± standard deviation in the CRF control group. BMD in the reference normal group was 0.269 ± 0.010 g/cm^2 ($N = 5$). *$P < 0.05$. (*B*) The gray bar shows the mean ± standard deviation in the CRF control group. BMD in the reference normal group was 0.363 ± 0.016 g/cm^2 ($N = 5$). *$P < 0.05$.

age with normal renal function (31 ± 5 mg/dL) ($P < 0.001$).

The groups treated with calcitriol or with the combined treatment ($E_2 - 15$ + calcitriol and $E_2 - 45$ + calcitriol) showed serum calcium levels that were significantly higher, whereas serum PTH levels were lower than those observed in the rest of the groups.

In the groups treated with the two doses of E_2 tested, alone or in combination with calcitriol, the serum levels of 17β-estradiol were within the range observed in the animals without ovariectomy (reference and control group). Furthermore, a proportional increase in the levels of 17β-estradiol with the dose of E_2 tested was observed (Table 1).

The groups treated with E_2 alone were able to only partially recover the uterus weight, whereas those treated with calcitriol alone had no effect at this level. Only the groups treated with E_2 + calcitriol achieved a uterus weight close to that of the rats with normal ovarian function (Fig. 1).

Bone mineral measurements

At the lumbar spine, the BMD values in the placebo group were significantly lower than those observed in the CRF control group ($P < 0.05$). Treatment with E_2 alone, at any of the doses tested, was not able to recover the bone loss that occurred after E_2 deprivation. Animals that received calcitriol alone or combined with E_2 reached BMD values similar to those of the control and reference groups (Fig. 2).

At the proximal tibia segment, animals treated with placebo or with E_2 at the lower dose showed low BMD values. In contrast, animals treated with $E_2 - 45$, with calcitriol, or with the combination of both ($E_2 - 15$ + calcitriol and $E_2 - 45$ + calcitriol), achieved BMD values that were within the range observed in the control and reference groups (Fig. 2).

At the distal tibia segment, only the group treated with $E_2 - 45$ + calcitriol achieved BMD values higher both in the rest of the treatment, control, and reference groups (0.27 ± 0.01 vs. 0.25 ± 0.01, $P < 0.001$).

DISCUSSION

The analysis of the different markers of estrogen replacement and the BMD measurements in our animal model with CRF + OVX showed that the combined therapy with E_2 + calcitriol was the most effective strategy in order to prevent the bone mass loss at lumbar and tibia sites.

Biochemical results showed that in the groups that received calcitriol or the combined treatment with E_2 + calcitriol, the levels of serum calcium were significantly higher, and the levels of PTH were significantly lower to those observed in the rest of the groups [5]. It is known that the anti-resorptive effect of calcitriol is due to a reduction of secondary hyperparathyroidism, a process that is mediated by the direct effect of the calcitriol on the gland or by an increase in the intestinal absorption of calcium [5, 12]. E_2 alone did not seem to have any effect neither on calcium nor on PTH serum levels. However, other studies have reported that E_2 has an effect that increases the intestinal calcium absorption, either

by a direct action of E_2 on renal 1α-hydroxylase [13] or on VDR [14] expression, or indirectly stimulating $1,25(OH)_2D$ renal synthesis through increments in PTH levels [13].

The treatment with E_2 alone (both doses) was unable to recover the bone loss at the lumbar spine. It could be argued that the estradiol dose was insufficient; however, the dose of 45 μg/kg/day extrapolated to humans can be considered in the range of normal-high dose [14, 15]. In contrast, treatment with E_2 at the dose of 45 μg/kg/day was able to recover the bone loss at the proximal tibia segment. In fact, at the proximal tibia, 45 μg/kg/day of E_2 was able to achieve BMD values similar to that observed in the groups with normal ovarian function. The greater response to E_2 observed at the tibia site, compared with the spine site, could be explained by the different proportion of cancellous and cortical bone in these two segments. It has been previously demonstrated that there is a greater loss of cancellous bone than of cortical bone in postmenopausal women [16] and in ovariectomized rats [17, 18]. A recent study on postmenopausal women [19] shows the positive effects of E_2 therapy on bone density at the lumbar spine using doses of 1 mg and 2 mg of E_2, which are proportional to the doses tested in our study. This different response in the clinical and experimental studies could also be explained by a lesser content of trabecular bone in the rat lumbar spine than in humans.

The animals which had received the combined treatment with E_2 + calcitriol, not only had a better BMD at the lumbar spine and proximal tibia, but also showed a better uterus weight gain than that observed in rats which had only received E_2, and similar to that observed in the groups with normal ovarian function (reference and control groups). This fact could be explained by a direct action not only of E_2, but also of calcitriol, on the uterus, possibly mediated by its receptor (VDR). In fact, there are studies that demonstrate the implication of the VDR in growth, bone formation, and female reproduction in adult age [20], including immunohistochemic evidence of the existence of this receptor in the uterus [21]. These facts support the hypothesis that the combined treatment could have a synergic effect not only on the uterus, but also on other tissues, such as bone, where the existence of the VDR and estrogen receptors α and β has been proven [22–24].

No differences at the distal tibia site after treatment were observed between the placebo and all of the other groups that received active drugs (data not shown). This is most likely explained by the fact that cortical bone is less affected by the ovariectomy than cancellous bone [16, 18]. Animals treated with high doses of E_2 + calcitriol had a significantly higher BMD at the distal tibia site than those observed in the rest of the treatment groups. It could be argued that this is due to a longitudinal bone growth, which is in accordance with the fact that rats do not close the growth plate; however, the areas of these segments are similar in all groups (data not shown). Previous studies have demonstrated greater effectiveness of this combined treatment on the cortical bone [25] compared with estrogen replacement alone. This fact seems to demonstrate that the combination of E_2 + calcitriol can have a direct effect on cortical bone, emphasizing the importance of the E_2 dose used, as the dose of 15 μg/kg/day of E_2 showed no effects at this level, neither alone nor combined with calcitriol.

In our study, a possible limitation of the beneficial effect of E_2 was the fact that the best results were obtained using a dose that could be considered close to the upper limit range in human replacement therapy [14, 15]. Taking into account the risk that estrogen therapy involves [26, 27], and the pharmacokinetics of E_2 in women with end-stage renal failure [28], the use of lower doses of this hormone would be more advisable. However, in our study, non-abnormal proliferative effects with normal-high doses of E_2 were observed in the uteri of these animals after two months of treatment. This is a very important finding, considering that this period of treatment in the rat is the equivalent of seven years of treatment in women. Histomorphometric studies currently being carried out in our laboratory will help us to determine with more precision the lowest effective dose of E_2 that should be used in the combined therapy [29].

The results obtained in our experimental model emphasize the usefulness of a replacement-combined therapy of E_2 + calcitriol in chronic renal failure with estrogen deficiency. Nonetheless, more studies to optimize the lower E_2 dose with greater efficiency are necessary in order to prevent the possible negative effects that the continuous administration of high doses of E_2 can involve.

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