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EFFECTS OF LONG-TERM TREATMENT WITH ESTRADIOL OR CLOMIPHENE CITRATE ON BONE MAINTENANCE, AND PITUITARY AND UTERINE WEIGHTS IN OVARIECTOMIZED RATS

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Summary—Long-term estrogen replacement therapy in postmenopausal women can bring relief to hot flushes and reduce loss of bone mass due to osteoporosis, however, such treatment often can cause uterine hyperplasia and other undesirable effects. This study compared changes in bone mineral content (BMC), uterine weight, pituitary weight and pituitary gonadotropin content in the ovariectomized rat model following treatment with estradiol (E_2) or two levels of clomiphene citrate (CC), an estrogen agonist/antagonist.

Groups ($n = 8$–12) of adult ovariectomized (OVX) rats were implanted with E_2 pellets (5 μg/day) or injected subcutaneously with CC at 1 mg/kg body wt (CC-1) or 5 mg/kg body wt (CC-5) twice weekly for 12 months. Placebo implanted OVX and intact (INT) female rats served as negative and positive controls, respectively. Following treatment, the uterus, pituitary gland and right femur were collected from each animal.

E_2 treatment increased ($P < 0.05$) uterine weight compared to all other treatment groups, while both CC doses increased uterine weight over the OVX group only (E_2, 0.24 ± 0.03; INT, 0.14 ± 0.01; CC-1, 0.06 ± 0.01; CC-5, 0.07 ± 0.01; and OVX, 0.02 ± 0.01 g per 100 g body wt). Pituitary weight was increased 15-fold ($P < 0.05$) by E_2 treatment over all other treatment groups (E_2, 65.7 ± 13.9; INT, 4.0 ± 0.5; CC-1, 3.3 ± 0.03; CC-5, 2.7 ± 0.02; and OVX, 2.9 ± 0.02 mg per 100 g body wt). Both E_2 and CC treatments reduced pituitary luteinizing hormone and follicle stimulating hormone content (μg/pit) to INT levels and were lower ($P < 0.05$) than OVX levels. Mean BMC of E_2, CC-1- or CC-5-treated rats was greater ($P < 0.05$) than that of either the INT or OVX groups, while INT animals had a higher BMC compared to OVX animals (E_2, 0.027 ± 0.003; CC-1, 0.026 ± 0.001; CC-5, 0.028 ± 0.001; INT, 0.021 ± 0.001; and OVX, 0.017 ± 0.001 g/cm per 100 g body wt). These data indicate that CC has the potential to reduce bone mineral loss without causing other undesirable effects, including uterine hyperstimulation, and thus needs to be further investigated.

INTRODUCTION

Cessation of ovarian function at menopause or due to ovariectomy in younger women results in the loss of ovarian steroidogenesis, particularly estradiol synthesis, and often leads to debilitating conditions known collectively as the postmenopausal syndrome. The majority of women (~80%) undergoing natural menopause may experience some form of vasomotor instability including flushing of the face, neck and chest ("hot flushes") and diaphoresis [1]; atrophy of the estrogen-dependent genitourinary system [2] and perhaps the most debilitating condition, progressive demineralization and reduction in bone mass (osteoporosis) [3].

These symptoms can be alleviated to varying degrees by estrogen replacement therapy. Treatment with exogenous estrogen dramatically reverses genitourinary atrophy and increases vaginal secretions [2]. Estrogen is known to have protective effects on the female skeleton and a causal relationship between estrogen deficiency and osteoporosis has been clearly established [4]. Although previous attempts to identify estrogen receptors in bone tissue had failed, recent data indicates that estrogen can act directly on osteoblasts through a specific receptor mediated mechanism. Evidence has been presented that estrogen receptors are present in normal human osteoblast-like cells [5] and in osteoblast-like osteosarcoma [6].

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Despite the beneficial effects of estrogen in relieving many of the postmenopausal symptoms, long-term therapy often results in undesirable effects including thrombic episodes, liver disease and development of estrogen-dependent tumors [7]. Estrogen-induced endometrial hyperplasia is considered to be a potentially premalignant condition and a significant number of such cases (up to 25%) can develop into endometrial adenocarcinoma depending upon the degree of hyperplasia and the duration of unopposed estrogen stimulation [8]. The addition of cyclic progesterone can reduce the hyperplastic condition [9], however, such a treatment regimen can reinitiate menstrual-like uterine bleeding which is viewed as an unacceptable alternative by many postmenopausal women. Because of the multifaceted problems associated with long-term estrogen therapy, it has been suggested that a pharmacologic analog of estrogen with fewer side-effects would be an ideal therapeutic agent [10].

A group of synthetic compounds exists which interacts with the estrogen receptor and possesses both estrogenic and antiestrogenic properties. Typically called "antiestrogens", these include clomiphene citrate (CC), tamoxifen, nafoxidine and chlorotrianisene. In general, these compounds are weak estrogen agonists and are estrogenic in the absence and antiestrogenic in the presence of circulating estrogens [11]. Both CC and tamoxifen have been shown to suppress the postmenopausal rise in gonadotropins and induce vaginal estrogenization in postmenopausal women without causing proliferative changes in the endometrium [12–14]. A recent study in intact and ovariectomized rats reported that tamoxifen had protective effects similar to estrogen on trabecular bone, but unlike estrogen was not uterotropic [15].

Although such compounds have potential therapeutic usefulness, results from prolonged treatment with estrogens and/or antiestrogens in an appropriate animal model are lacking. The present investigation was designed to document changes in uterine weight, pituitary weight, pituitary gonadotropin content and bone mineral content (BMC) of ovariectomized (OVX) adult rats following long-term treatment with estradiol (E_2) or CC.

EXPERIMENTAL

Adult female Sprague–Dawley rats (60 days old) were either left intact (INT) or were bilaterally ovariectomized (OVX). Following a recovery period of 7 days, OVX animals ($n = 8$–12) were assigned at random to four treatment groups. Animals were provided free access to pelleted rat feed and water. One group of OVX rats was subcutaneously implanted with slow release E_2 pellets (Innovative Research of America, Toledo, OH, U.S.A.) designed to deliver 5 μg E_2/day. Each of two groups of OVX rats were injected subcutaneously twice weekly with CC in doses of either 1 mg/kg body wt (CC-1) or 5 mg/kg body wt (CC-5). One group of OVX rats implanted with placebo pellets served as a negative control group while a group of untreated INT rats served as positive controls. Pellets were replaced at 60-day intervals and animals were sacrificed at the end of 12 months of continuous treatment.

At sacrifice the uterus, pituitary gland and right femur from each animal were removed and weights recorded. Pituitaries were stored frozen at $-80°C$ prior to hormone analysis. Each femur was cleaned of all extraneous soft tissue and then stored in a desiccator prior to BMC determination. The BMC at mid-shaft was determined by single energy photon absorptiometry (Model SP2 Rectilinear Scanner, Lunar Radiation Corp.) in air using a 1.0 mm collimator at a scan speed of 0.25 mm/s, with bones positioned on a custom made plexiglass platform. This method has been shown to be highly reproducible and precise ($\pm 2\%$ c.v.) according to a previously described report [16].

Pituitary luteinizing hormone (LH) and follicle stimulating hormone (FSH) contents were determined using specific radioimmunoassays kits (NIADDK, National Institutes of Health, U.S.A.) as previously described [17].

Because of differing weight gains between treatment groups, uterine weight, pituitary weight and BMC were adjusted per 100 g body wt prior to statistical analysis. All data were analyzed by least square analysis of variance and group means were compared using Student–Newman–Keul's procedure for significant differences among means between groups for each parameter examined.

RESULTS

Effects on uterine weight

Treatment of ovariectomized rats with E_2 over a 12 month period increased ($P < 0.05$) mean uterine weight (0.24 ± 0.03 g/100 g body

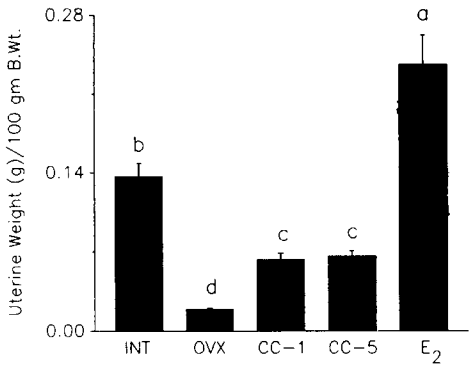

Fig. 1. Mean (±SEM) uterine weight of different groups following 12 months of treatment. Different superscripts denote differences ($P < 0.05$).

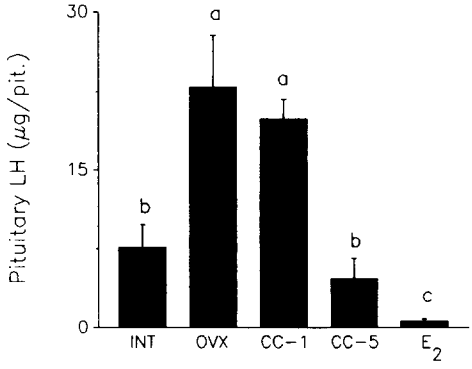

Fig. 3. Mean (±SEM) pituitary LH content of different groups following 12 months of treatment. Different superscripts denote differences ($P < 0.05$).

wt) approx. two-fold over INT animals, four-fold over either group of CC-treated animals and 12-fold over the OVX rats (Fig. 1). Treatment with either dose of CC resulted in a significant increase ($P < 0.05$) in mean uterine weight over the placebo-treated OVX control animals but was lower ($P < 0.05$) compared to that of the INT or E_2-treated animals.

Effects on pituitary

Mean pituitary weight of the E_2-treated group (65.7 ± 13.9 mg/100 g body wt) was greater ($P < 0.05$) than that of all other groups (Fig. 2). Mean pituitary weights of all other groups were similar ($P > 0.05$) and ranged between 2.7 ± 0.2 mg/100 g body wt (CC-5) to 4.0 ± 0.5/100 g body wt for the INT group.

Treatment with E_2 significantly reduced pituitary LH content (0.61 ± 0.21 μg/pit) compared to that of all other groups (Fig. 3). While the higher clomiphene dose (CC-5) reduced post-ovariectomy pituitary LH content to levels lower than that of INT animals (4.64 ± 1.96 vs 7.61 ± 2.21 μg/pit, respectively), the lower dose (CC-1) did not reduce pituitary LH content (19.90 ± 1.90 μg/pit) and was similar to that of the OVX group (22.97 ± 4.83 μg/pit).

Effects of various treatments on pituitary FSH content were similar to those described for LH content (Fig. 4). E_2 treatment resulted in pituitary FSH content similar ($P > 0.05$) to that of INT animals (4.13 ± 1.29 vs 2.78 ± 1.31 μg/pit). Treatment with either the low (CC-1, 16.59 ± 3.54 μg/pit) or high dose (CC-5, 15.86 ± 2.72 μg/pit) of CC reduced ($P < 0.05$) FSH content compared to that of the OVX group (43.42 ± 3.72 μg/pit), but CC at either dose was not as effective as E_2 in decreasing pituitary FSH.

Bone mineral content

Mean BMC of the right femur in CC-1-(0.026 ± 0.001 g/cm per 100 g body wt), CC-5-(0.028 ± 0.001 g/cm per 100 mg body wt) and E_2-(0.027 ± 0.003 g/cm per 100 g body wt) treated groups were similar ($P > 0.05$) and were maintained at a significantly ($P < 0.05$) higher level compared to that of the INT (0.021 ± 0.001 g/cm per 100 g body wt) or the OVX

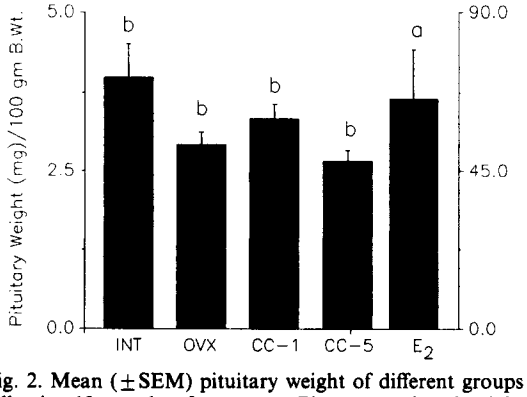

Fig. 2. Mean (±SEM) pituitary weight of different groups following 12 months of treatment. Please note that the right hand ordinate is for the E_2 group only. Different superscripts denote differences ($P < 0.05$).

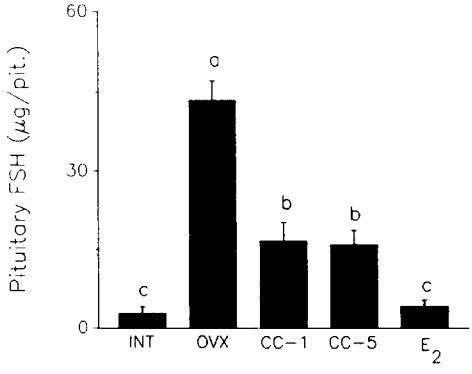

Fig. 4. Mean (±SEM) pituitary FSH content of different groups following 12 months of treatment. Different superscripts denote differences ($P < 0.05$).

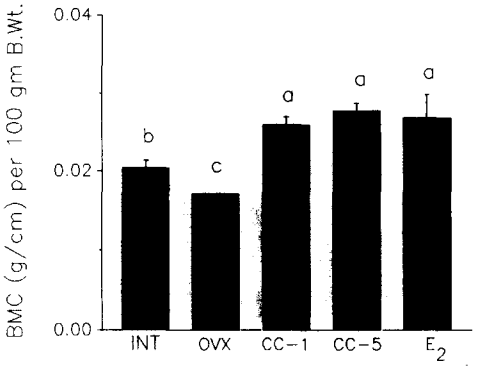

Fig. 5. Mean (±SEM) femur bone mineral content of different groups following 12 months of treatment. Different superscripts denote differences ($P < 0.05$).

(0.017 ± 0.001 g/cm per 100 g body wt) groups (Fig. 5). The mean BMC of the INT group, although lower than that of the E_2- or CC-treated groups, was higher ($P < 0.05$) than that of the OVX group.

DISCUSSION

Data obtained from this study clearly establishes that long-term estrogen therapy of OVX rats causes uterine hyperstimulation beyond that normally observed in INT animals. Ovariectomy of rats without estrogen supplementation caused uterine atrophy. However, when animals were treated with either dose of CC the uterine weight increased two-fold over that of OVX animals, indicative of estrogenic stimulation, but did not become hypertrophied. Clomiphene citrate and some other antiestrogens primarily stimulates the endometrial epithelium, whereas E_2 causes hypertrophy and hyperplasia of both endometrial and myometrial components [11, 18]. The present data confirms CC to be a partial estrogen agonist, capable of at least partially maintaining normal uterine weight.

Long-term treatment of OVX rats with E_2 resulted in a marked increase in pituitary weight while suppressing the post-ovariectomy increase in pituitary LH and FSH. Prolonged treatment of rats with estrogen is known to induce pituitary hyperplasia or tumors accompanied by increased serum prolactin concentrations [19]. Although not as effective in primates [20], the high concentration of circulating estrogen during human pregnancy can result in a significant proliferation of pituitary lactotrophs and elevated levels of circulating prolactin [21]. The significant increase in pituitary weight observed in this study was associated with a concomitant increase in serum prolactin concentration (data not shown), and was probably the result of E_2-induced pituitary lactotroph hyperplasia. Although not as effective as E_2, the higher dose of clomiphene (CC-5) reduced pituitary LH content to less than INT levels and significantly reduced pituitary FSH content from that observed in the untreated OVX control animals.

The present study provides evidence that long-term treatment with CC was at least as effective as E_2 in preventing bone loss by maintaining BMC. Ovariectomy and loss of ovarian estrogen, or the normal aging process are known to cause cortical and trabecular bone loss. A normal age-related loss in BMC was also observed in the INT group of animals. Treatment with estrogen or tamoxifen have been shown to maintain bone mass in OVX rats [15]. In a previous study prevention of osteoporosis and maintenance of cortical bone thickness and total body calcium by chronic CC treatment has also been reported [22]. The present data support these earlier findings.

Taken together, the data obtained in this study indicate that long-term E_2 treatment of OVX rats prevented osteoporosis due to loss of BMC but caused uterine hyperstimulation and pituitary hyperplasia. Use of CC as an alternative treatment for the same duration prevented bone loss without any adverse uterine or pituitary consequences. These results suggest that CC warrants further evaluation as an alternative to long-term estrogen therapy in postmenopausal women.

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REFERENCES

1. Bates G. W.: On the nature of hot flushes. *Clin. Obstet. Gynec.* **24** (1981) 231–241.
2. Hammond C. B. and Maxon W. S.: Current status of estrogen therapy for the menopause. *Fert. Steril.* **37** (1982) 5–25.
3. Raisz L. G.: Local and systemic factors in the pathogensis of osteoporosis. *New Engl. J. Med.* **318** (1988) 818–827.
4. Nordin B. E. C., Need A. G., Morris H. A. and Horowitz M.: New approaches to the problem of osteoporosis. *Clin. Orthop.* **200** (1985) 181–197.
5. Eriksen E. F., Colvard D. S., Berg N. J., Graham M. L., Mann K. G., Spelsberg T. C. and Riggs B. L.: Evidence of estrogen receptors in normal human osteoblast-like cells. *Science* **241** (1988) 84–86.
6. Komm B. S., Terpening C. M., Benz D. J., Graeme K. A., Gallegos A., Korc M., Greene J. L., O'Malley B. W. and Haussler M. R.: Estrogen binding, receptor mRNA, and biologic response in osteoblast-like osteosarcoma cells. *Science* **241** (1988) 81–84.

7. Hammond C. B., Jelovsek F. R., Lee K. L., Creasman W. T. and Parker R. T.: Effects of long-term estrogen replacement therapy II. Neoplasia. *Am. J. Obstet. Gynec.* **133** (1979) 537–547.
8. Gola H.: Hyperplasia of the endometrium. In *The Uterus* (Edited by A. T. Hertzig and M. R. Abel). Williams & Wilkins, Baltimore (1973) pp. 260–275.
9. Wentz A. C.: Assessment of estrogen and progestin therapy in gynecology and obstetrics. *Clin. Obstet. Gynec.* **20** (1977) 461–482.
10. Parfitt A. M.: Trabecular bone architecture in the pathogenesis and prevention of fracture. *Am. J. Med.* **82** (Suppl. 1B) (1987) 68–72.
11. Clark J. H. and Markaverich B. M.: The agonistic-antagonistic properties of clomiphene: a review. *Pharmac. Ther.* **15** (1982) 467–519.
12. Hashimoto T., Miyai K., Izumi K. and Kumahara Y.: Effect of clomiphene citrate on basal and LRH-induced gonadotropin secretion in postmenopausal women. *J. Clin. Endocr. Metab.* **42** (1976) 593–594.
13. Kistner R. W., Lewis J. L. and Steiner G. J.: Effects of clomiphene citrate on endometrial hyperplasia in the premenopausal female. *Cancer* **19** (1966) 115–126.
14. Natrajan P. K. and Greenblatt R. B.: Clomiphene citrate. In *Induction of Ovulation* (Edited by R. B. Greenblatt). Lea & Febiger, Philadelphia (1979) pp. 35–76.
15. Turner R. T., Wakley G. K., Hannon K. S. and Bell N. H.: Tamoxifen inhibits osteoclast-mediated resorption of trabecular bone in ovarian hormone-deficient rats. *Endocrinology* **122** (1988) 1146–1150.
16. Kiebzak G. M., Smith R., Howe J. C. and Sacktor B.: Bone mineral content in the senescent rat femur: an assessment using single photon absorptiometry. *J. Bone Miner. Res.* **3** (1988) 311–317.
17. Brown J. L. and Chakraborty P. K.: Characterization of the effects of clomiphene citrate on reproductive physiology in male rats of various ages. *Acta Endocr.* **118** (1988) 437–443.
18. Clark J. H. and Guthrie S. G.: Agonistic and antagonistic effects of clomiphene citrate and its isomers. *Biol. Reprod.* **25** (1981) 667–672.
19. Clifton K. H. and Meyer R. K.: Mechanism of anterior pituitary tumor induction by estrogen. *Anat. Rec.* **125** (1956) 65–81.
20. Bethea C. L.: Stimulatory effect of estrogen on prolactin secretion from primate pituitary cells, cultured on extracellular matrix and in serum-free medium. *Endocrinology* **115** (1984) 443–451.
21. Jacobs L. S. and Mariz I. K.: A mixed heterologous radio-immunoassay for human prolactin. *J. Clin Endocr. Metab.* **34** (1972) 484–490.
22. Beall P. T., Misra L. K., Young R. L., Spjut H. J., Evans H. J. and LeBlanc A.: Clomiphene protects against osteoporosis in the mature ovariectomized rat. *Calc. Tiss. Int.* **36** (1984) 123–127.