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Original Research

Effects of 2-[3-estrone-*N*-ethyl-piperazine-methyl] tetracycline (XW630) on osteoporosis in ovariectomized rats[1]

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KEY WORDS XW630; tetracyclines; estradiol; osteoporosis; ovariectomy; femur; radioimmunoassay; uterus; vaginal smears

ABSTRACT

AIM: To study the effects of 2-[3-estrone-*N*-ethyl-piperazine-methyl] tetracycline (XW630) on experimental osteoporosis in ovariectomized (OVX) rats. METHODS: Serum estradiol (E_2) content and bone 1-carboxyglutamic acid-containing protein (BGP) content were measured by radioimmunoassay. With undecalcified bone section and tetracycline intraperitoneal labeling, the bone static and dynamic data were studied in right femur samples. RESULTS: After treatment with XW630 2.5 mg·kg^{-1}, serum BGP content increased by 75.7 % but there was no change in serum E_2 content and uterus weight compared with OVX rats. Compared with OVX rats, the static data of trabecular bone volume/total tissue volume, trabecular bone volume/sponge bone volume, and mean trabecular plate density were enhanced after treatment with XW630 for 13 wk. The dynamic data of single-labeled surface, double-labeled surface, trabecular osteoid surface, and bone formation rate in tissue level in XW630 group were increased and osteoid maturation period was shortened. CONCLUSION: XW630 enhanced bone activation frequency and increased trabecular connectivity, stability, and strength. XW630 stimulated bone formation and inhibited bone resorption with no effect on reproductive system.

INTRODUCTION

The protective effects of estrogen on bone are well established from *in vivo* observation. Estrogen replacement is effective in preventing postmenopausal osteoporosis by reversing the reduction in circulating estrogen levels that occur at menopause[1].

In an attempt to attenuate the effect of estrogens on the reproductive system and enhance its effect on bone, ZHENG Hu, *et al* synthesized an estrogen-derived drug, 2-[3-estrone-*N*-ethyl-piperazine-methyl] tetracycline (XW630), which is a new conjugate of tetracycline and estrone.

Addicted to bone, tetracycline directs the drug to the bone tissue and increases the effect of estrone on the treatment of osteoporosis. On the other hand, tetracycline stimulates bone formation by itself[2,3]. The estrone, also produced by the premenopausal ovary, declines across menopause, but the reduction in its circulating levels is considerably less than that of E_2[4]. The influence of estrone on the reproductive system is only 10 % of E_2's. In acute toxicological tests, no death was reported in test mice after continuous treatment with XW630 6 g·kg^{-1}·d^{-1} for 7 d (to be published). In the study reported here, we investigated the effects of XW630 on the experimental osteoporosis in ovariectomized rats.

MATERIALS AND METHODS

Drugs and reagents XW630 (yellow crystal, purity > 98 %) was synthesized by School of Pharmacy, West China University of Medical Sciences, Chengdu,

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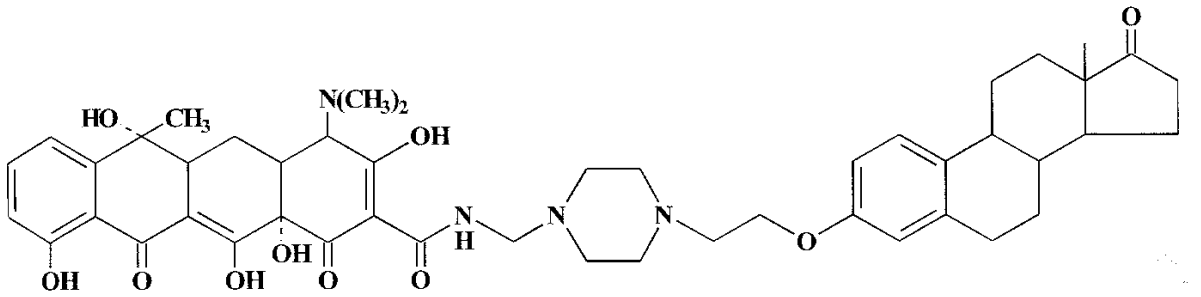

2-[3-Estrone-*N*-ethyl-piperazine-methyl] tetracycline (XW630)

China (CP 93110919, 6；93110939, 1；USP 8338505). XW630 was dissolved in Me_2SO with stock concentration of 0.1 mol·L^{-1}. Tetracycline and estrone obtained from Sigma were dissolved in absolute ethanol and stored at a final concentration of 0.1 mol·L^{-1}.

Rats Twelve month-old female Wistar rats with a mean body weight of (350 ± *s* 15) g were purchased from the Animal Institute of Chinese Academy of Medical Sciences (Grade Ⅱ, Certificate № 01-3055). Except for the rats which were operated without the oophorectomy (sham-operated), others were abdominally ovariectomised[2]. Rats were divided into 4 groups of 12 each：① sham-operated, ② ovariectomized (OVX)；③ estrone treated：one week after the operation, the drug was orally given at a dose of 1.0 mg·kg^{-1}·d^{-1} for 13 wk, six times a week；④ XW630, 2.5 mg·kg^{-1}·d^{-1}. The time course was the same as in the ③ group.

Vaginal smear From the 2nd to the 12th week (for every two weeks), a glass pipette was put into the vagina of every rat to obtain the cells. Cells were collected and washed with 0.9 % NaCl and resuspensed in PBS. Every time, the test was taken continuously for 6 d a week. In estrus, there were a lot of keratinocytes, but there were lots of polynuclear leukocytes and some epithelial cells in inter-estrus.

Measurement of serum E_2 and BGP concentration After 13-week treatment, all rats were decapitated and the blood was taken for the measurement of serum ^{125}I-E_2 concentration and the ^{125}I-BGP content by the method of radioimmunoassay (kits purchased from Beijing North Institute of Biological Technics).

Histomorphometric analysis[6,7] All rats were given tetracycline 30 mg·kg^{-1} intraperitoneally at 15 and 4 d, respectively, before sacrifice. After the rats were decapitated, the right femurs were removed and saved in 70 % ethanol solution for 2 d, stained by 0.5 % Villanueva Osteochrome Bone Stain for 5 d and then destained and dehydrated with sequential concentration changes (70 %, 95 %, and 100 %) of ethanol solution and xylene. They were then embedded in methymethacrylate. The bones were cut into longitudinal section of 100 μm thickness and then coverslipped for static and dynamic histomorphometric studies.

Bone histomorphometric parameters of the femur terminal sections were measured with epifluorescent/light microscope (Olympus, BH-2). The static data are TBV, TTV, SBV, trabecular bone surface (TBS), mean trabecular plate thickness (MTPT), mean trabecular plate spacing (MTPS), and MTPD which were performed with light microscope in undecalcified sections. The dynamic data are Sfract (s, d), TOS, Svf, distance between the double labels (DDL), osteoid maturation period (OMP), mineral apposition rate (MAR), and mean osteoid seam width (MOSW) which were performed with epifluorescent microscope in Villanueva stained sections.

Statistical analysis All the histomorphological data were performed with student-Newman-Keuls procedure for two-factor analyses of variance (SPSS software box, USA1993). A significance level of $P < 0.05$ was used for all comparisons. Results were expressed as $\bar{x} \pm s$.

RESULTS

Effects of XW630 on body weight, uterine weight, and serum E_2 and BGP concentration In this experiment, the rat body weight in all groups remained at baseline control levels. The uterus weight decreased sharply after ovariectomy. When OVX rats were treated with estrone, the uterus weight increased almost reaching sham-operated rat level. The uterus weight of OVX rats changed only slightly after treatment with XW630 (Tab 1).

We tested the vaginal smear to detect the estrus cycle

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Tab 1. Effects of XW630 on body weight, uterine weight, and serum levels of estradiol. $n = 12$. $\bar{x} \pm s$. $^{b}P < 0.05$, $^{c}P < 0.01$ *vs* sham-operated. $^{f}P < 0.01$ *vs* OVX. $^{h}P < 0.05$, $^{i}P < 0.01$ *vs* estrone.

	Sham-operated rat	OVX rat	estrone treated OVX rat	XW630 treated OVX rat
Body weight/g	350 ± 15	361 ± 28	360 ± 21	361 ± 34
uterus weight/mg	110 ± 30	30 ± 2^{c}	90 ± 3^{f}	40 ± 3^{ci}
serum E_2/ng·L^{-1}	149 ± 62	57 ± 23^{c}	379 ± 87^{cf}	73 ± 33^{i}
BGP/mg·L^{-1}	4.9 ± 1.8	3.7 ± 1.2^{b}	4.3 ± 1.8	6.5 ± 3.3^{bfh}

of rats. In the sham-operated rats, the changes took place 1 to 1.2 times a week. A 0.3 to 0.5 cycle per week was found in the OVX rats, which was remarkably lower than that of the control. However, in the OVX rats treated with estrone, the estrus cycle was increased by 66.7 %. In OVX rats treated with XW630, 0.8 to 0.9 cycle was found every week, which was higher than that of OVX rats, but lower than that of control rats.

In the rats treated with XW630, the uterus weight was the same as that of OVX. This result indicated that XW630 had no effect on the estrogen target organ, which was distinguishable from estrone.

There was no significant change in the serum E_2 concentration and the uterus wet weight in the rats after treatment with XW630. In the estrone group, the serum E_2 concentration increased while the uterus wet weight enhanced.

The XW630 also affected the serum BGP content (increased by 75.7 % *vs* OVX, $P < 0.05$). But the estrone did not change the serum BGP content.

Effects of XW630 on histomorphometry

Compared with that of sham-operated rats, the static data of TBV/TTV, TBV/SBV and MTPD of OVX rats were reduced by 38 %, 30 %, and 33 %, respectively, while the MTPS increased by 37 % (Tab 2).

After treatment with XW630, the TBV/TTV, TBV/SBV, MTPT, and MTPD were enhanced by 38 %, 25 %, 11 %, and 13 %, respectively compared with that of OVX rats. All of these data were close to those of sham-operated rats. However, after treatment with estrone, there were no statistical differences between OVX rats and the other estrone-treated rats.

The changes in bone dynamic histomorphometry are shown in Tab 3.

In the OVX rats, OMP was increased by 107 % and MAR decreased by 16 % as compared to the sham-operated rats. The single- and double-labeled surface slightly decreased by 46.2 % and 25 %, respectively. No differences in MOSW and MLT were found between the sham-operated group and the OVX rats.

After treatment with estrone, single- and double-labeled surface enhanced by 104.9 % and 416.7 %, respectively, and OMP shortened by 71.8 % as compared to the OVX rats. These results suggest that estrone stimulates bone formation. In this study, the effects of XW630 on MAR, bone formation rate, tetracycline labeled surfaces, and OMP were more than those of estrone.

Tab 2. Static data of XW630 on bone histomorphometry of right femurs in OVX rats. $n = 6$. $\bar{x} \pm s$. $^{b}P < 0.05$ *vs* sham-operated (control). $^{e}P < 0.05$ *vs* OVX. $^{h}P < 0.05$ *vs* estrone.

	Sham-operated	OVX rat	Estrone treated OVX rat	XW630 treated OVX rat
TBV/TTV (%)	19 ± 5	12 ± 8^{b}	13 ± 5	16 ± 6^{eh}
S/V (mm^2/mm^3)	33 ± 4	31 ± 3	32 ± 3	28 ± 2
TBV/SBV (%)	21 ± 5	15 ± 3^{b}	17 ± 4	19 ± 6^{e}
MTPT (μm)	61 ± 6	64 ± 6	64 ± 6	72 ± 6^{eh}
MTPD (/mm^2)	3.5 ± 0.7	2.3 ± 0.5^{b}	2.7 ± 0.6^{b}	2.6 ± 0.8^{b}
MTPS (μm)	238 ± 61	380 ± 76^{b}	328 ± 102^{b}	345 ± 121

TBV：trabecular bone volume；TTV：total tissue volume；SBV：sponge bone volume；TBS：trabecular bone surface；MTPT：mean trabecular plate thickness；MTPD：mean trabecular plate density；MTPS：mean trabecular plate spacing.

Tab 3. Dynamic data of XW630 on bone histomorphometry of right femur in OVX rats. $n=6$. $\bar{x}\pm s$.
$^bP<0.05$, $^cP<0.01$ *vs* sham-operated. $^fP<0.01$ *vs* OVX. $^iP<0.01$ *vs* estrone.

	Sham-operated	OVX rat	Estrone treated OVX rat	XW630 treated OVX rat
Sfract (s)/%	7.8 ± 4.6	4.1 ± 3.5^b	8.4 ± 1.8	13.3 ± 4.5^f
Sfract (d)/%	0.8 ± 0.9	0.6 ± 0.3^b	3.1 ± 2.2	3.0 ± 1.8
Sfract (d + 1/2s)/%	4.7 ± 3.2	2.6 ± 2.1^b	7.1 ± 3.1^f	9.7 ± 2.8^f
DDL/μm	8.3 ± 1.4	7.1 ± 1.2	8.2 ± 1.0	9.5 ± 0.8^f
TOS/%	4.4 ± 3.7	2.6 ± 1.5^b	1.4 ± 1.1^f	2.4 ± 1.5
MOSW/μm	3.6 ± 1.5	3.5 ± 1.3	3.8 ± 1.1	4.5 ± 0.8^f
MAR/μm·d^{-1}	0.64 ± 0.08	0.48 ± 0.08^b	0.58 ± 0.07	0.73 ± 0.06^f
MLT/d	6.1 ± 2.2	7.2 ± 2.5	6.7 ± 1.9	6.8 ± 1.5
Svf/μm·d^{-1}	0.05 ± 0.01	0.04 ± 0.01^b	0.06 ± 0.03	0.16 ± 0.09^{cfi}
OMP/d	169 ± 94	351 ± 175^b	99 ± 44^{cf}	83 ± 54^{cf}

Sfract (s)：single-labeled surface；Sfract (d)：double-labeled surface；DDL：distance between the double labels；TOS：trabecular osteoid surface；MOSW：mean osteoid seam width；MAR：mineral apposition rate；MLT：mineralization lag time；Svf：bone formation rate in tissue level；OMP：osteoid maturation period.

DISCUSSION

In our studies, we demonstrated that the treatment with XW630 or estrone resulted in the same degree of effect on the vagina cells of OVX rat. However, XW630 2.5 mg·kg^{-1} did not influence the weight of uterus, while estrone 1.0 mg·kg^{-1} increased the uterus weight dramatically with a high serum E_2 concentration. These data suggest that XW630 did not affect the reproductive system. BGP is an important protein for Ca^{2+}-phosphate combination and bone matrix mineralization. Thus, serum BGP elevation indicates bone formation. In this experiment, BGP content increased significantly in OVX rats treated with XW630, suggesting that XW630 promoted bone formation.

The static data of TBV/TTV and TBV/SBV represent bone volume of trabecula, while S/V, MTPT, MTPT, and MTPS, represent trabecular architecture[8]. After ovariectomy, the TBV/TTV and TBV/SBV declined while MTPD was lower than that of sham-operated. These results indicate that the bone volume and trabecular density in diaphysis decreased with oophorectomy. The dynamic data of Sfract (s) and Sfract (d) represent bone activation frequency, while the TOS, MAR, and Svf, represent trabecular connectivity. The Sfract (s), Sfract (d), TOS, MAR, and Svf of OVX rats were less than those of sham-operated indicating that the bone activation frequency was reduced. After the treatment with XW630, the Sfract (s), Sfract (d), TOS, Svf, and MAR increased significantly while OMP shortened showing that XW630 enhanced bone activation frequency. The connectivity of trabecular bone and activation frequency of bone in OVX rats increased after the treatment with XW630 was initiated. We also found that TBV/TTV and TBV/SBV were higher than the control levels and MTPD, MTPT, and MTPS were improved by some degree in XW630 group. All these effects of XW630 were stronger than those of estrone. These results demonstrat that XW630 not only increased the bone mass but also improved trabecular spatial architecture and enhanced stability and strength of bone.

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XW630 对去卵巢大鼠骨质疏松的作用[1]

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关键词　XW630；四环素类；雌二醇；骨质疏松；卵巢切除术；股骨；放射免疫测定；子宫；阴道涂片

目的：研究 XW630 对去卵巢大鼠骨质疏松的影响. **方法：**血清 E_2 和骨钙素（BGP）浓度用放射免疫测定法. 骨组织计量学用四环素内标法. **结果：**去卵巢后，血清 E_2 水平下降 61.9 %，子宫减轻 72.7 %，动情次数减少 63.6 %. XW630 治疗 13 周后，血清 BGP 浓度增加 75.7 %，动情次数略增加，但低于假手术组，血清 E_2 浓度及子宫重量无明显变化. 与 OVX 组相比，XW630 组骨组织计量学指标 TBV/TTV，TBV/SBV 和 MTPD 增加；Sfract（s），Sfract（d），TOS 和 Svf 增加，OMP 缩短. **结论：**XW630 增加骨激活频率、骨小梁连接性、稳定性和张力. 表明 XW630 促进骨形成、抑制骨吸收，对生殖系统无影响.

（责任编辑　刘俊娥）