

1 DHEA administration activates local bioactive androgen metabolism in cancellous site of tibia  
2 of ovariectomized rats

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13 **Running title:** DHEA and local androgen metabolism in bone tissue

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1 **Abstract** It is not known whether local androgen metabolism is involved in the mechanisms  
2 underlying the dehydroepiandrosterone (DHEA) administration-induced improvement of bone  
3 mineral density (BMD) in an estrogen deficiency state. The aim of the present study was to  
4 clarify whether DHEA administration would improve local androgen metabolism and BMD in  
5 cancellous site of tibia of ovariectomized (OVX) rats. Twenty-two female rats, 6 weeks old,  
6 were randomized into three groups: sham-operated rats, OVX control rats, and OVX rats that  
7 received DHEA treatment. DHEA was administered intraperitoneally at 20 mg DHEA/kg body  
8 weight for 8 weeks. The concentrations of free testosterone and dihydrotestosterone (DHT) in  
9 cancellous site of tibia did not change as a result of ovariectomy, while the DHT concentration  
10 increased following DHEA administration. We revealed that DHEA administration improved the  
11 reduction of  $17\beta$ - and  $3\beta$ -hydroxysteroid dehydrogenases and clearly reversed the reduction of  
12  $5\alpha$ -reductase type 1 and 2 and androgen receptor in the cancellous site of tibia of OVX rats.  
13 DHEA administration suppressed estrogen deficiency relative to the decrease in the cancellous  
14 BMD, which was positively associated with local DHT concentration. These findings indicate  
15 that DHEA administration enhances local bioactive androgen metabolism in the cancellous tibia  
16 of young OVX rat, suggesting that local DHT may play a part in the DHEA  
17 administration-induced improvement of cancellous BMD.

18 **Key words:** estrogen deficiency, BMD, DHEA, androgen metabolism

## 1 **Introduction**

2 Before natural menopause, primary ovarian insufficiency defined as the premature cessation  
3 of ovarian function or menstrual dysfunction due to disordered eating or the female athlete triad  
4 syndrome is associated with severe risks for bone health [1, 2]. The ovariectomized (OVX) rat  
5 is a useful animal model for examining actions of sex steroids on bone. In young aged rats,  
6 ovariectomy induces the increases in both longitudinal and radial growth, as well as significant  
7 loss of trabecular bone [3, 4]. Although it is well known that the trabecular bone loss caused by  
8 ovariectomy is attributable to estrogen deficiency, it is not clear what actions other gonadal  
9 hormones have for bone metabolism in the estrogen deficiency state.

10 Dehydroepiandrosterone (DHEA), secreted mainly from the adrenal gland and ovary,  
11 plays a critical physiological role for maintaining steroidogenesis by being used as the available  
12 precursors converted to testosterone and estrogens in the various peripheral tissues such as the  
13 bones, liver, brain, skeletal muscles, and so on [5]. DHEA concentration in the blood decreases  
14 following ovariectomy in animals [6, 7]. On the other hand, DHEA replacement improves bone  
15 mineral density (BMD) in OVX animals [6, 7]. However, the mechanisms by which DHEA  
16 induces the BMD increase are largely unknown.

17 In osteoblast cells (OBs), DHEA can be converted to testosterone and further metabolized  
18 to dihydrotestosterone (DHT), which has the physiological strong function [8-10] and is known

1 to be the most powerful androgen because its higher affinity for androgen receptor (AR) than  
2 that of testosterone [11]. DHEA also increases the transcription of AR in OBs [7]. In particular,  
3 DHT prevents cancellous bone loss in OVX rats [12], and AR knockout female mice have a  
4 decreased amount of cancellous bone as well [13]. Thus, a decrease in circulating DHEA level  
5 caused by ovariectomy could deactivate the transcription of local androgen-related steroid  
6 enzymes in bone tissue, probably leading to decreased cancellous bone mass. However, it is not  
7 clear whether DHEA administration would activate the local androgen-related steroid enzymes  
8 or hormones in vivo during a period of rapid bone loss caused by estrogen deficiency.

9 We previously showed that estrogen deficiency reduced the cancellous BMD of tibia but  
10 not the cortical BMD in OVX rats [14]. Moreover, DHEA administration apparently improved  
11 the cancellous bone osteopenia in OVX rats [6]. Accordingly, we hypothesized that DHEA  
12 administration would activate the local androgen-related steroidogenesis with lessening the  
13 reduction of cancellous BMD of tibia caused by estrogen deficiency. In the present study, we  
14 examined androgen levels of free testosterone and DHT, steroidogenesis-related enzymes of  
15  $17\beta$ -hydroxysteroid dehydrogenase ( $17\beta$ -HSD),  $3\beta$ -HSD,  $5\alpha$ -reductase type 1 and 2, and AR,  
16 and BMD in a cancellous site of the tibia in OVX rats after 8 wks of DHEA administration.

17

## 18 **Materials and methods**

1 **Experimental animals and feeding protocol.** Twenty-two female Sprague-Dawley rats, 6  
2 weeks old, were randomized into three groups: sham-operated rats (Sham Control; SC, n=5),  
3 OVX control rats (OC, n=8), and OVX rats with DHEA treatment (OD, n=9). The OVX groups  
4 were ovariectomized via the dorsal route, and the sham-operated rats were operated on without  
5 the ovaries being removed. During the period of the experiment, all rats were fed a diet with  
6 1.05% calcium and 1.01% phosphate purchased from CLEA Japan (CE-2, CLEA Japan, Inc.,  
7 Japan). DHEA dissolved in sesame oil was administered to the OD group intraperitoneally at 20  
8 mg DHEA/kg body weight for 8 weeks beginning one week after ovariectomy while the SC and  
9 OC groups were treated with vehicle only (sesame oil, 0.5 ml) [15]. Rats were not treated with  
10 DHEA or vehicle every fourth day (i.e., they were treated for 3 consecutive days). The rats were  
11 kept in individual cages (15×25×19.5cm) and allowed access to food and distilled water *ad*  
12 *libitum*. Food consumption and body weight gain were measured every second day. The room  
13 temperature was kept at 24±1 °C, the humidity at 50±5%. Fluorescent lights were on from 8:00  
14 a.m. to 8:00 p.m.. Animal care and experimental procedures were approved by the Animal  
15 Experimental Committee of the University of Tsukuba.

16

17 **Measurement of bone mineral density.** The left tibia of each rat was isolated by dissection and  
18 freed from any muscle and connective tissue. Thereafter, bone mineral content (BMC) and bone

1 mineral density (BMD) values for the whole tibia were measured by Dual-energy X-ray  
2 absorptiometry (DXA; Aloka DCS-600R instrument). We studied the proximal one-fifth of the  
3 tibia, including the epimetaphyseal region representing the cancellous sites, and middle  
4 one-fifth of the tibia representing the cortical diaphyseal region [16].

5

6 **Immunoblot analysis.** Western blot analysis of the cancellous site (proximal one-fifth of the  
7 tibia) of the right tibia of each rat was performed according to the procedure described in our  
8 previous paper with minor modifications [17]. After completely removing marrow and blood,  
9 the tibia fragment was homogenized by grinding the tissue in a mortar on dry ice with 10  
10 volumes of 20 mM Tris-HCL (pH 7.8), 2 mM EDTA, 300 mM NaCl, 2 nM DTT, 2% NP 40,  
11 0.2% SDS, 0.2% sodium deoxycholate, 0.5 mM PMSF, 60 µg/ml aprotinin, and 1 µg/ml  
12 leupeptin. The homogenate was gently rotated for 30 min at 4 °C and then centrifuged at 12,000  
13 g for 15 min at 4 °C. The protein concentration of the resulting supernatant was determined by  
14 protein assay rapid kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Samples (50 µg  
15 protein) were subjected to heat denaturation at 96 °C for 7 min with Laemmli buffer. Western  
16 blot analysis for the detection of 17β- and 3β-HSD, 5α-reductase type 1 and 2, and AR was  
17 performed according to the procedure described in our previous report [17, 18]. Briefly, each  
18 sample was separated on an SDS-polyacrylamide gel (10 %) and then transferred to a

1 polyvinylidene difluoride (PVDF, Millipore, Billerica, MA, USA) membrane. The membrane  
2 was then incubated in blocking buffer of 3% skimmed milk in phosphate-buffered saline  
3 containing 0.1% Tween-20 (PBS-T) for 1 h at room temperature followed by incubation with  
4 primary antibodies, a polyclonal anti-17 $\beta$ -HSD and anti-3 $\beta$ -HSD (1:1000 dilution, Santa Cruz  
5 Biotechnology), a polyclonal anti-5 $\alpha$ -reductase type 1 and 2 (1:1000 dilution, Abnova  
6 Corporation, Taipei, Taiwan), a polyclonal anti-AR (1:1000 dilution, Santa Cruz Biotechnology),  
7 and a monoclonal anti- $\beta$ -actin (1:1000 dilution, Santa Cruz Biotechnology), for 12 h at 4°C.  
8 Subsequently, the membrane was washed with PBS-T three times, and incubated for 1 h at room  
9 temperature with a horseradish peroxidase (HRP)-conjugated secondary antibody, either an  
10 anti-rabbit IgG (1:2000 dilution, Amersham Biosciences, Piscataway, NJ, USA) or an  
11 anti-mouse IgG (1:2000 dilution, Amersham Biosciences).. After this reaction, the membrane  
12 was washed with PBS-T five times. Finally, binding was detected by chemiluminescence with  
13 the ECL Plus system (Amersham Life Sciences), and the membrane was exposed to Hyper film  
14 (Amersham Biosciences).

15

16 **Sandwich-enzyme immunoassay (EIA).** Concentrations of E<sub>2</sub> and DHEA in serum and free  
17 testosterone and DHT in bone tissue were determined using a sandwich EIA kit (free  
18 testosterone: IBL International GMBH, Flughafenstrasse, Hamburg, Germany; E<sub>2</sub>: Cayman

1 Chemical, Ann Arbor, MI, U.S.A.; DHEA: Assay Designs, Inc., Ann Arbor, MI, U.S.A.; DHT:  
2 IBL International GMBH, Flughafenstrasse, Hamburg, Germany). The protein samples of bone  
3 tissue for the EIA analysis were the same as those for the western blot analysis. All techniques  
4 and materials used in this analysis were in accordance with the manufacturers' protocols. The  
5 sensitivities of intra- and inter-assay coefficients of variation for the hormone assays were as  
6 follows: at dose of free testosterone (62 pg/ml for intra-assay precision; 76 pg/ml for inter-assay  
7 precision), 4.7% and 9.3%, respectively; at dose of E<sub>2</sub> (102 pg/ml for intra- and inter-assay  
8 precision), 13.0% and 8.2%, respectively; at dose of DHEA (1039 pg/ml for intra-assay  
9 precision; 1093 pg/ml for inter-assay precision), 5.8% and 6.5%, respectively; at dose of DHT  
10 (237 pg/ml for intra-assay precision; 281 pg/ml for inter-assay precision), 11.4% and 12.1%,  
11 respectively.

12  
13 **Statistics.** All the data are expressed as mean  $\pm$  SE. Statistical analysis was carried out by  
14 analysis of variance (ANOVA) followed by Fisher's F-test for multiple comparisons. A  
15 significance level of  $p < 0.05$  was used for all comparisons. Association between BMD and  
16 serum E<sub>2</sub> or local DHT levels was determined by the Pearson correlation test. All statistical  
17 treatments were done using the Stat View 5.01 software (SAS Institute, Inc., Cary, NC, USA,  
18 2000-2001).



1 <The position of Table 1>

## 2 **Results**

3 Initial body weight did not differ among the three groups. The final body weight, body  
4 weight gain, and food intake of the OVX groups were significantly higher than those of the SC  
5 group (Table 1). The final body weight and body weight gain of the OD group were  
6 significantly lower than those of the OC group, but there were no differences in food intake. The  
7 serum concentration of DHEA and E<sub>2</sub> in the OC group was found to be significantly lower  
8 compared to that of the SC group and significantly higher in the OD group than in the OC group  
9 (Table 1). There were no differences in serum free testosterone concentration between the SC  
10 and OC groups, but the concentration was significantly higher in the OD group than in the OC  
11 group.

12 <The position of Figure 1>

13 The BMD of the tibial proximal metaphysis (cancellous site) in the OC group was found  
14 to be significantly lower compared to that in the SC group, but this decrease was significantly  
15 attenuated in the OD group. On the other hand, there were no differences among the 3 groups in  
16 the BMD of the tibial diaphysis (cortical-abundant region) (Fig. 1).

17 <The position of Figure 2>

18 In the tibial proximal metaphysis, free testosterone concentration did not differ among the  
19 3 groups (Fig. 2). On the other hand, local DHT concentration was significantly higher in the

1 OD group than in the OC group, whereas there was no difference between the SC and OC  
2 groups (Fig. 2).

3 The local concentration of DHT was also significantly correlated with the BMD of the tibial  
4 proximal metaphysis in the OVX groups ( $r = 0.706, p < 0.05$ ). The local DHT concentration  
5 was significantly associated with the serum concentration of DHEA ( $r = 0.936, p < 0.001$ ), free  
6 testosterone ( $r = 0.933, p < 0.001$ ), or  $E_2$  ( $r = 0.817, p < 0.01$ ) in the OVX groups. Also, there  
7 was a significant correlation between the BMD of tibial proximal metaphysis and the serum  
8 concentration of DHEA ( $r = 0.741, p < 0.01$ ), free testosterone ( $r = 0.754, p < 0.01$ ), or  $E_2$  ( $r =$   
9  $0.685, p < 0.05$ ) in the OVX groups. In the correlation analysis, we used only 11 samples (n=6  
10 for the OC group and n=5 for the OD group) because of hemolysis in blood samples.

11 The protein expressions of  $17\beta$ - and  $3\beta$ -HSD,  $5\alpha$ -reductase type 1 and 2, and AR were  
12 significantly lower in the OC group than in the SC group and significantly higher in the OD  
13 group than in the OC group (Fig. 3).

14 *<The position of Figure 3>*

## 15 **Discussion**

16 The present study results confirmed that DHEA administration improved the decrease in  
17 the cancellous BMD of tibia caused by estrogen deficiency. The present study demonstrated that  
18 DHEA administration increased the local DHT concentration in the cancellous site of tibia of

1 OVX rats. We also revealed that DHEA administration improved the reduction of  $17\beta$ - and  
2  $3\beta$ -hydroxysteroid dehydrogenases and clearly reversed the reduction of  $5\alpha$ -reductase type 1  
3 and 2 and androgen receptor in the cancellous site of tibia of OVX rats. Moreover, the  
4 cancellous BMD of tibia was positively correlated with the local DHT concentration in OVX  
5 rats. These results suggest that local bioactive androgen metabolism plays a role, at least in part,  
6 in the mechanism underlying DHEA administration-induced improvement of cancellous bone  
7 loss caused by estrogen deficiency.

8         The present study demonstrated that estrogen deficiency clearly reduced the activation of  
9 the androgen-related steroidogenic enzymes. An increase in DHEA enhances the transcriptions  
10 of the androgen-related steroidogenic enzymes in OBs [7-9]. We previously also demonstrated  
11 that androgen-related hormones could be locally synthesized by improving the transcriptions of  
12 the androgen-related steroidogenic enzymes from DHEA in muscle tissues as well [17]. Thus,  
13 we assumed in the present study that the decrease in circulating DHEA level by OVX might  
14 have partly deactivated the transcriptions of the androgen-related steroidogenic enzymes in the  
15 bone tissue. However, it is unclear whether estrogen deficiency reduced the activation of the  
16 androgen-related steroidogenic enzymes through DHEA, directly or indirectly or both.

17         In vitro studies using OBs demonstrated that DHEA can be metabolized to testosterone by  
18  $17\beta$ - and  $3\beta$ -HSD and further metabolized to DHT by the  $5\alpha$ -reductase type 1 and 2 enzymes,

1 binding to the AR [7-9]. These results mostly corresponded to the results of the present study in  
2 a cancellous site of the tibia in vivo that DHEA administration increased the DHT concentration  
3 and the steroidogenesis-related enzymes in  $17\beta$ - and  $3\beta$ -HSD, the  $5\alpha$ -reductase type 1 and 2,  
4 and AR. However, in the present study, DHEA administration did not induce any increase in the  
5 local free testosterone concentration. Free testosterone is metabolized to DHT only through the  
6  $5\alpha$ -reductase enzymes [11, 19]. Actually, in the present study, the expression of  $5\alpha$ -reductase  
7 type 1 and 2 was greatly increased to the level of sham-operated rats. Moreover, testosterone  
8 can be metabolized not only to DHT but  $E_2$  by p450arom enzyme activity [7]. Thus, the  
9 transient increase in testosterone might have occurred, but the testosterone was soon  
10 metabolized to synthesize DHT and/or  $E_2$ .

11 The present study results showed that DHEA administration clearly reversed the reduction  
12 of AR induced by estrogen deficiency as well. Wang et al. [7] reported that DHEA increases the  
13 transcription of AR in OBs. AR knockout mice have a decreased amount of cancellous bone  
14 [13]. DHT also has the capacity to prevent ovariectomy-induced cancellous bone loss [12]. On  
15 the other hand, the inhibitor of  $5\alpha$ -reductase does not appear to have a bone-sparing effect [20].  
16 This implies that the increase in local DHT concentration with the activation of AR by DHEA  
17 administration might play a critical role in maintaining bone-sparing in an estrogen deficiency  
18 state.

1       The present study showed the significant correlation between the BMD and local DHT  
2 concentration in the site of cancellous bone loss caused by estrogen deficiency. Especially, the  
3 marked increase in serum free testosterone concentration by DHEA administration was found  
4 and the serum free testosterone concentration was highly correlated with the local DHT  
5 concentration. Thus, we cautiously considered that the increase in the serum free  
6 testosterone concentration by DHEA administration may, at least in part, induce the  
7 increase in local DHT level, resulting in the increase in the cancellous BMD. On the  
8 other hand, the present study also demonstrated that DHEA administration resulted in a  
9 substantial rise in serum E<sub>2</sub> concentration which was also positively correlated with the  
10 cancellous BMD of tibia. The increase in serum E<sub>2</sub> concentration prevents severe bone loss  
11 caused by estrogen deficiency [7]. Thus, it could not be excluded that the increase in serum E<sub>2</sub>  
12 concentration by DHEA administration would have a sufficient bone sparing effect irrespective  
13 of the local DHT level. Therefore, to evaluate if parts of the DHEA effect was dependent on  
14 local DHT level, a further study is strongly needed to demonstrate if the DHEA effect would be  
15 reduced by co-treatment with androgen receptor antagonist or if the DHEA effect would be  
16 sustained by co-treatment with estrogen receptors or aromatase antagonist.

17       Although the present study results confirmed that DHEA prevented bone loss caused by  
18 estrogen deficiency and improved local bioactive androgen metabolism, DHEA concentration in

1 serum was approximately 13 times higher in OVX rats treated with DHEA than in  
2 sham-operated control rats. Future study should focus on the dose-dependent effect of DHEA  
3 administration on the relation between BMD and local bioactive androgen metabolism.  
4 Furthermore, the present study results confirmed that the estrogen deficiency reduced the  
5 cancellous BMD but not the cortical BMD as reported previously [14]. Moreover, DHEA  
6 administration prevented the cancellous BMD loss but did not affect the cortical BMD.  
7 However, the BMD measurement by DXA in the present study did not allow the details of bone  
8 parameters such as cortical width and periosteal or endosteal circumferences. Thus, the  
9 parameters should be examined in detail using by the methods of pQCT,  $\mu$ CT, or bone  
10 morphometry in further studies.

11 In conclusion, the present study results demonstrated for the first time that in a cancellous  
12 site of the tibia, DHEA administration increased the DHT concentration and improved the  
13 reduction of the androgen steroidogenesis-related enzymes caused by estrogen deficiency.  
14 Furthermore, DHEA administration suppressed the decrease in the cancellous BMD of tibia  
15 induced by estrogen deficiency, which was significantly associated with the local DHT  
16 concentration. These results suggested that improvement of local bioactive androgen  
17 metabolism by DHEA administration could contribute to preventing the loss of cancellous BMD  
18 in estrogen deficiency state.

1

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1 **Figure legends**

2

3 **Figure 1.** Bone mineral density of tibial proximal metaphysis (A) and diaphysis (B).

4 The proximal one-fifth of the tibia, including the epimetaphyseal region representing the

5 cancellous sites, and middle one-fifth of the tibia, representing the cortical diaphyseal

6 region. SC, Sham-operated control group; OC, Ovariectomy-operated control group;

7 OD, Ovariectomy group with DHEA treatment. \*\*\*  $p < 0.001$  vs. SC; ##  $p < 0.01$  vs.

8 OC. Values are expressed as the means  $\pm$  SE.

9

10 **Figure 2.** Concentrations of free testosterone (A) and DHT (B) in the proximal

11 metaphysis of the tibia (trabecular bone). The tissue concentrations were normalized for

12 total protein. SC, Sham-operated control group; OC, Ovariectomy-operated control

13 group; OD, Ovariectomy group with DHEA treatment. ###  $p < 0.001$  vs. OC. Values are

14 expressed as the means  $\pm$  SE.

15

16 **Figure 3.** Concentrations of 17 $\beta$ -HSD protein (A), 3 $\beta$ -HSD protein (B), 5 $\alpha$ -reductase

17 type 1 protein (C), 5 $\alpha$ -reductase type 2 protein (D), and androgen receptor protein (E) in

18 the proximal metaphysis of the tibia (trabecular bone). The tissue concentrations were

1 normalized for total protein. SC, Sham-operated control group; OC,

2 Ovariectomy-operated control group; OD, Ovariectomy group with DHEA treatment.

3 \*\*\*  $p < 0.001$  vs. SC; ###  $p < 0.001$  vs. OC. Values are expressed as the means  $\pm$  SE.

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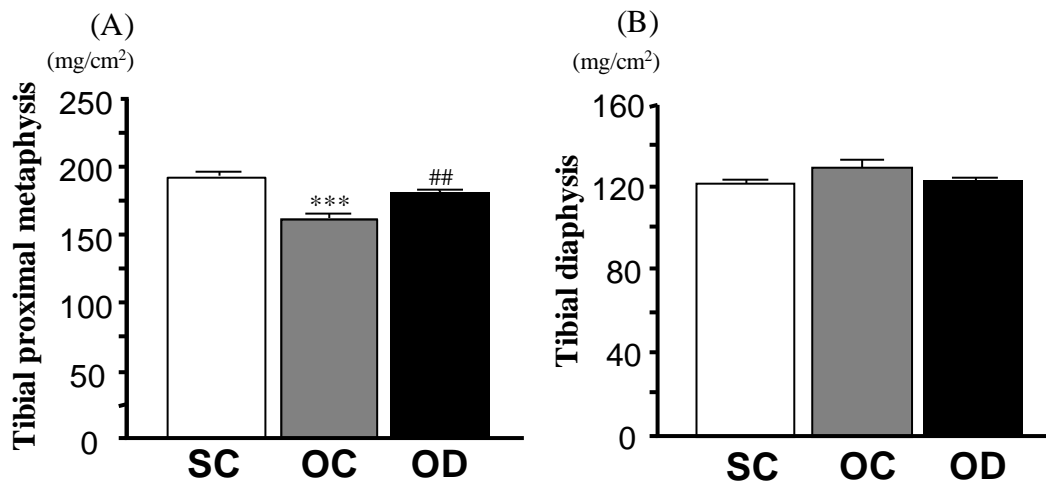
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1 Figure 1.



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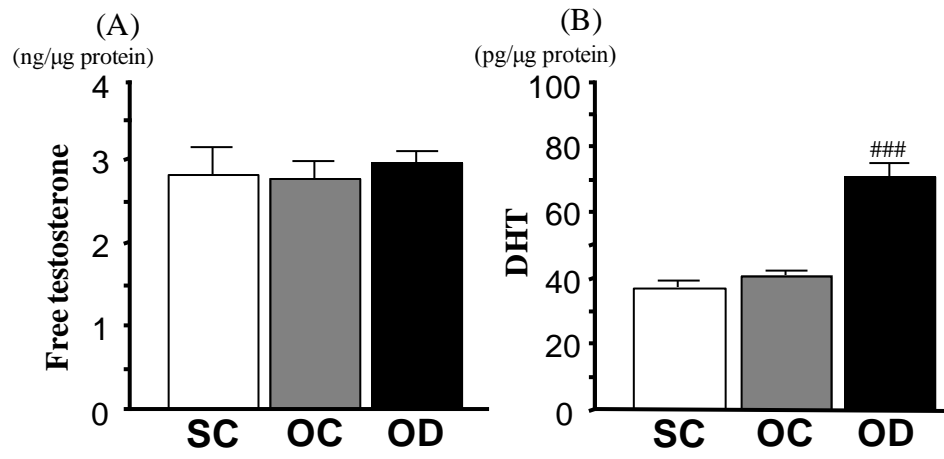
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1 Figure 2.



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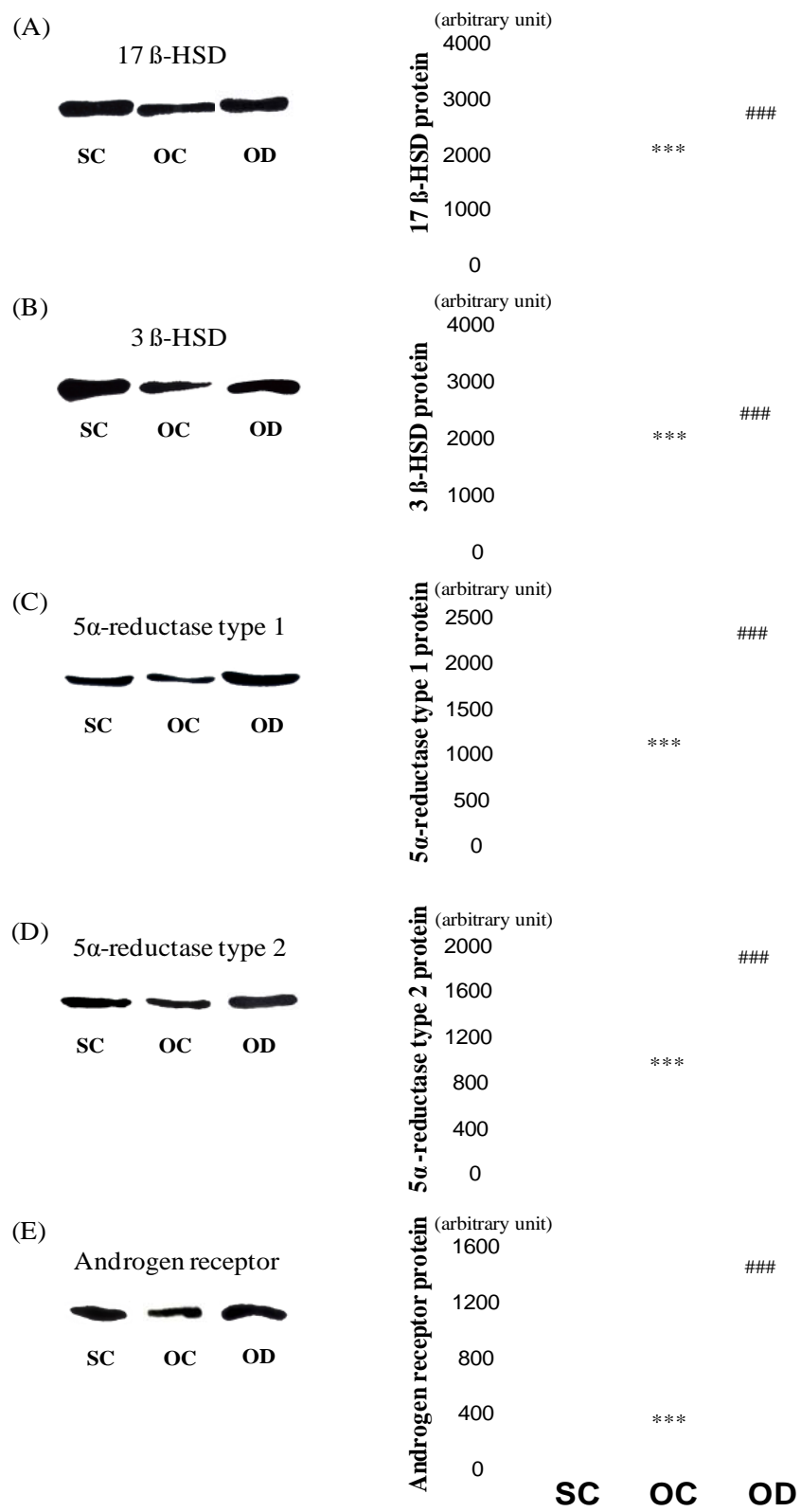
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1 Figure 3.



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**Table 1. Body weight, food intake, and steroid hormones in serum**

	Final body weight (g)	Body weight gain (g/day)	Food intake (g/day)	DHEA (ng/ml)	E <sub>2</sub> (pg/ml)	Free testosterone (pg/ml)
SC	274.8 ± 5.8	2.0 ± 0.1	15.4 ± 0.4	2.7 ± 0.2	35.8 ± 5.9	2.9 ± 0.2
OC	340.5 ± 6.3***	3.2 ± 0.1***	17.7 ± 0.2***	1.1 ± 0.2**	13.4 ± 1.3*	1.2 ± 0.1
OD	309.1 ± 6.8##	2.6 ± 0.1 <sup>#</sup>	17.4 ± 0.5	35.0 ± 0.6###	53.1 ± 10.0###	71.7 ± 3.1###

Data show mean ±SE. SC, Sham-operated control group; OC, Ovariectomy-operated control group; OD, Ovariectomy-operated group with DHEA treatment. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  vs. SC; #  $p < 0.05$ , ##  $p < 0.01$ , ###  $p < 0.001$  vs. OC.

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