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Asymmetric Dimethylarginine Is Not Involved in Ovariectomy-Induced Osteopenia in Rats

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Previous studies have indicated that the plasma concentration of nitric oxide synthase inhibitor, asymmetric dimethylarginine (ADMA), was increased in postmenopausal women. In the study reported here, we tested the relationship between the decrease of bone mineral density (BMD) and ADMA concentration in ovariectomized (OVX) rats. Ovariectomized rats at 8 months of age were treated with 17β-estradiol (10 or 30 μg/kg of body weight/day, s.c.) or L-arginine (300 mg/kg/day, i.p.) for 12 weeks (n = 10 for each group). Pre- and posttreatment total BMD, posttreatment plasma nitrite/nitrate and ADMA concentrations, and posttreatment BMD in the lumbar part of the spine (L4–L6), femurs, and tibias were examined. Ovariectomy caused a significant decrease in several BMD indexes, which was reversed by estrogen treatment ($P < 0.05$). Plasma nitrite/nitrate concentration was significantly decreased in OVX rats, but was restored by estrogen treatment ($P < 0.05$). There were no differences in the plasma concentration of ADMA in OVX or estrogen-treated rats. L-Arginine had no effect on plasma nitrite/nitrate concentration and BMD in OVX rats. These results suggest that ovariectomy does not influence the plasma concentration of ADMA, and that ADMA is not involved in ovariectomy-induced osteopenia in rats.

It has been suggested that nitric oxide (NO) participates in regulation of bone formation and bone resorption (25). It has been reported that the decrease of NO synthesis contributes to development of osteoporosis in vitro and in vivo, and NO donors, such as nitroglycerin, increase bone mineral density (BMD) in patients or rats that have been oophorectomized (OVX) (1, 10, 26).

Nitric oxide is synthesized from L-arginine by nitric oxide synthase (NOS). Recently, it was found that asymmetric dimethylarginine (ADMA), one of the methylated arginine compounds, can inhibit NO synthesis, suggesting that ADMA is an endogenous mechanism that regulates NO synthesis (7). Interestingly, plasma concentration of ADMA is increased in postmenopausal women (9, 18, 24). Our recent work indicated that BMD was decreased concomitantly with an increase in endogenous ADMA concentration in aged rats, suggesting that this increase may be associated with development of osteoporosis (15). Therefore, in the study reported here, we tested whether ADMA is involved in development of osteopenia in OVX rats. Since L-arginine, the precursor of NO, can competitively antagonize the effect of NOS inhibitors, endogenous or exogenous (13), we also tested the effect of L-arginine on BMD in OVX rats.

Materials and Methods

Animal preparation and experimental protocol. Female Sprague-Dawley rats, aged 8 months, were purchased from the Animal Center of Xiang-Ya School of Medicine. Before and after surgery, animals were housed in metabolism cages and allowed ad libitum access to water and a standard rat pellet diet (Animal Center of Xiang-Ya School of Medicine). Room temperature, relative humidity, ventilation, and lighting were 23 ± 1°C, 55 ± 5%, 15 air changes/h, and light cycle from 6 a.m. to 6 p.m., respectively. The study was performed in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (1986 ed.) and was approved by the Central South University Veterinary Medicine Animal Care and Use Committee. Rats were monitored and maintained to be free of the following pathogens: *Salmonella* sp., *Listeria monocytogenes*, *Yersinia pseudotuberculosis* and *Y. enterocolitica*, pathogenic dermal fungi, *Pasteurella multocida* and *P. pneumotropica*, *Bordetella bronchiseptica*, *Mycoplasma pulmonis*, *Corynebacterium kutscheri*, *Bacillus piliformis*, epidemic hemorrhagic fever virus, Sendai virus, rat parvovirus, sialodacryoadenitis virus, pneumonia virus of mice, murine adenovirus, *Toxoplasma gondii*, *Taenia* sp., *Hymenolepis* sp., *Syphacia, aspiculuris tetraptera*, *Trichosomoides crassicauda*, and ectoparasites. Animals under anesthesia with sodium pentobarbital (60 mg/kg of body weight, i.p.) underwent bilateral ovariectomy. The sham surgery group underwent the same anesthesia procedure, but without ovariectomy. The drug-treated group was treated with 17β-estradiol (10 or 30 μg/kg/day, five days per week, s.c.; Sigma Chemical Co., St. Louis, Mo.) or L-arginine (300 mg/kg/day, five days per week, i.p.). The drugs were dissolved in a vehicle containing 97% sesame oil and 3% ethanol. Meanwhile, the sham surgery group and the OVX group were given the vehicle. Body weight of animals was recorded before surgery and at monthly intervals throughout the experiment. After 12 weeks of treatment, the rats were sacrificed under anesthesia, then plasma was obtained and stored at –70°C for 17β-estradiol, ADMA, and NO measurements, and the uterus from

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each rat was obtained and weighed. Samples from the left femur and tibia and the lumbar part of the spine, from L4 to L6, were resected, and connective tissues were carefully eliminated.

Measurement of bone mineral density (15). Bone mineral density was measured by use of dual energy X-ray absorptionetry (DXA) with a Hologic QDR 4500A fan beam x-ray bone densitometer (Hologic Inc., Waltham, Mass.). The preparations were positioned supine on the table and were orientated perpendicular to the pass of the x-ray beam, using a scan step of 0.254 mm and a scan speed of 6.077 mm/sec. Total BMD before and after treatment and BMD of full bone in the lumbar part of the spine (L4–L6), the femurs, and the tibias after treatment were determined. The BMD was expressed in grams per square centimeter.

Determination of ADMA concentration. Samples were deproteinated by addition of 0.2 ml of methanol, and were allowed to settle for 10 min after sufficient intermixing. Then the mixture was centrifuged at 12,000 ×*g* for 10 min. The supernatant was used for measurement of ADMA by use of a liquid chromatography- mass spectrometry (LC-MS) system. The LC-MS was performed using a Shimadzu LCMS-2010 liquid chromatograph-mass spectrometer with electrospray positive ion (ESI). Separation of the analyte was achieved using a 2.1 × 150-mm, 5-µm Hypurity C18 column (Thermo Hypersil-Keystone, Bellefonte, Pa.) with a flow rate of 0.2 ml/min. The column oven temperature was set at 35°C. The mobile phase was acetonitrile:water (96:4) with 0.1% acetic acid and 0.1% trifluoroacetic acid. A 5-µl sample was injected. Instrument settings for mass spectrometry were as follows: ESI selective ion mode (ADMA: M/Z = 203; Arg: M/Z = 175), probe voltage of 4.5 kV, curved desolvation line voltage of 50 V, detector voltage of 1.6 kV, nebulizer gas flow of 4.5 L/min, CDL temperature of 250°C, and block temperature 200°C.

Determination of nitrite/nitrate concentration. Plasma nitric oxide concentration was determined indirectly the content of nitrite and nitrate. Nitrite was converted to nitrate by *Aspergillus* nitrite reductase, and total nitrate concentration was measured using the Griess reagent. Absorbance at 540 nm was determined using a spectrophotometer.

Measurement of 17β-estradiol concentration. Plasma concentration of 17β-estradiol was analyzed using a ^{125}I radioimmunoassay technique (Dongya Immunity Technology Institution, Beijing, China) according to the manufacturer's instructions.

Statistical analysis. Results are expressed as mean ± SEM. All data were analyzed by using one-way analysis of variance and the Student Newman Keuls *t* test. Significance was set at $P < 0.05$.

Results

As indicated (Fig. 1A and 1B), plasma 17β-estradiol concentration and uterine weight were significantly decreased in OVX rats. Estrogen replacement therapy increased 17β-estradiol concentration and uterine weight. Treatment with L-arginine had no effect on 17β-estradiol values and uterine weight.

There were no significant differences in body weight before surgery. Ovariectomy caused a significant increase in body weight, which was prevented by treatment with estrogen at a dosage of 10 or 30 µg/kg/day ($P < 0.05$). L-Arginine treatment had no effect on body weight in OVX rats (Table 1).

Table 2 illustrates the changes among groups in total BMD, before and after treatment, and BMD in the lumbar part of the spine (L4–L6), the femurs, and the tibias of rats after treatment.

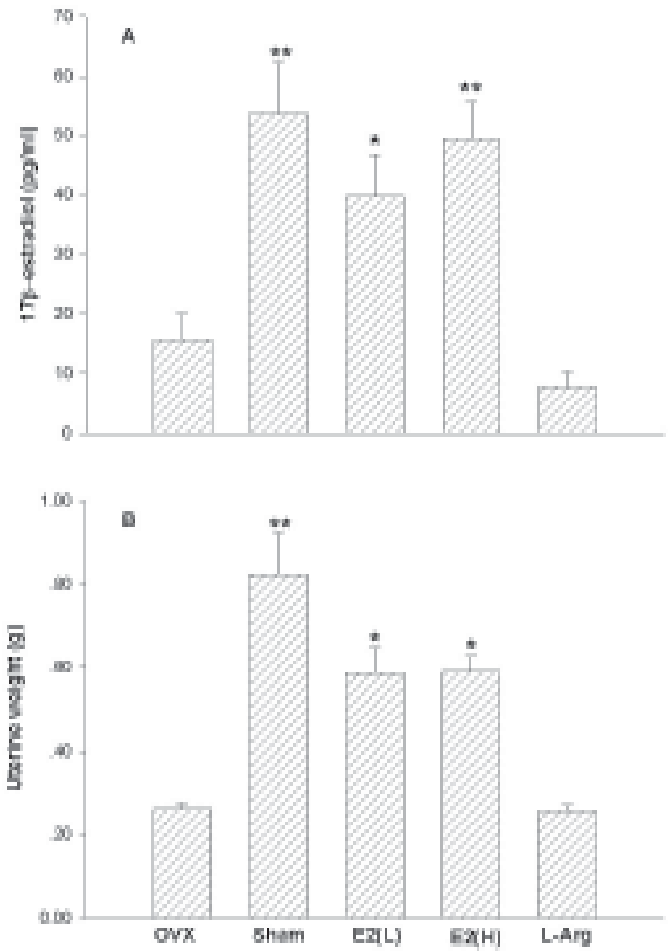

Figure 1. Effect of 17β-estradiol or L-arginine on plasma concentrations of 17β-estradiol (A) and uterine weights (B). OVX = ovariectomized; E2 (L) = ovariectomy plus 17β-estradiol (10 µg/kg/d); E2 (H) = ovariectomy plus 17β-estradiol (30 µg/kg/d); L-Arg = ovariectomy plus L-arginine (300 mg/kg/d); Sham = sham surgery plus vehicle. Values are mean ± SEM (n = 10/group). $^{*}P < 0.05$ versus OVX; $^{**}P < 0.01$ versus OVX.

There were no differences in total BMD before the experiment; however, three months after ovariectomy, several BMD indexes decreased accordingly, and were reversed by estrogen ($P < 0.05$), but not L-arginine treatment.

Plasma nitrite/nitrate concentration was significantly decreased in OVX rats; 17β-estradiol, but not L-arginine treatment, increased nitrite/nitrate concentration (Fig. 2A). There were no differences in ADMA concentration among the groups (Fig. 2B).

Discussion

It has been reported that surgically induced or natural menopause leads to osteoporosis, and estrogen treatment can delay or reverse this disease in vivo and in vitro (19, 21). Results of numerous studies have suggested that the effect of estrogen may be partly mediated by NO. It has been reported that the NO concentration in plasma was decreased after depletion of estrogen and was increased after estrogen replacement therapy in humans and animals (6, 20). In the presence of N^{G}-nitro-L-arginine methylester, an inhibitor of NOS, estrogen was totally ineffective in reversing the bone loss in OVX rats (27). Administration of nitroglycerin can restore ovariectomy-induced bone loss in rats or

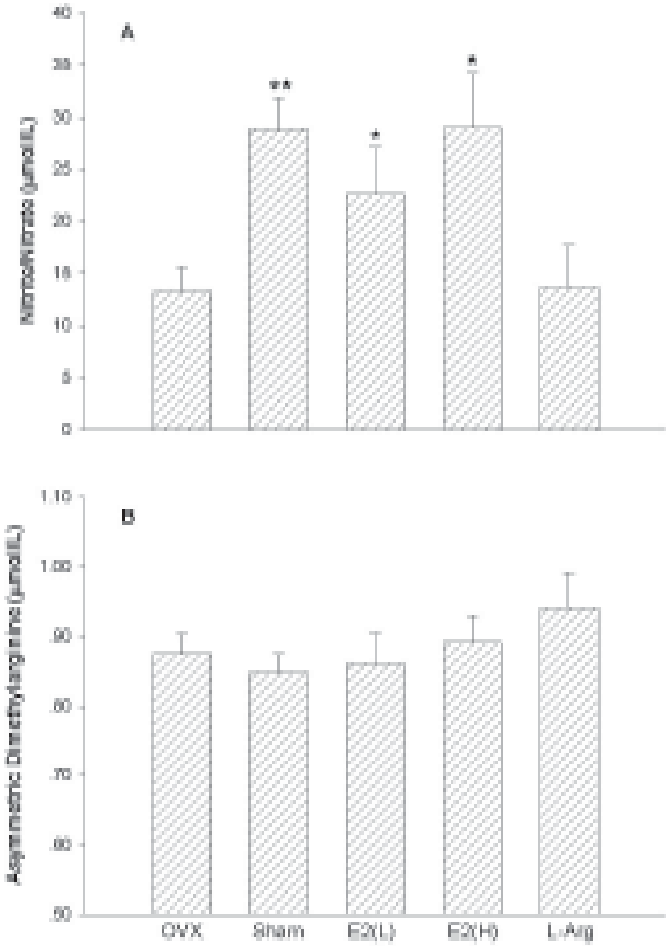

Figure 2. Effect of 17β-estradiol or L-arginine on plasma concentrations of nitrite/nitrate (A) and asymmetric dimethylarginine (B). *See* Fig. 1 for key.

increase BMD in postmenopausal women (10, 26).

It is known that NO is synthesized from its precursor, L-arginine, by the enzyme NOS. There is evidence to suggest that estrogen can increase production of NO by activation of NOS (17). Most recently, it was reported that the plasma concentration of ADMA, an endogenous inhibitor of NOS, was high in postmenopausal women and that administration of 17β-estradiol caused a significant decrease in plasma ADMA concentration (9, 18, 24). In mouse bone marrow-derived mesenchymal stem cells, ADMA significantly inhibited osteoblast differentiation and function (28). Our recent work indicated that the increased content of endogenous ADMA may be associated with development of osteoporosis since BMD was decreased concomitantly with an

Table 1. Effect of 17β-estradiol (E2) or L-arginine (L-Arg) on mean body weight (g) in rats (n = 10/group)

Variable	Pre-treatment	1 month	2 months	3 months
OVX	317 ± 7	359 ± 12††	383 ± 15††	405 ± 18††
Sham	309 ± 9	322 ± 10*	332 ± 12*	341 ± 15*
E2 (L)	314 ± 6	340 ± 9†	345 ± 10*†	351 ± 13*†
E2 (H)	318 ± 9	331 ± 11	339 ± 11*	348 ± 12*
L-Arg	324 ± 10	366 ± 17†	385 ± 20†	398 ± 22††

OVX = ovariectomized; E2 (L) = ovariectomized plus 17β-estradiol (10 μg/kg/d); E2 (H) = ovariectomized plus17β-estradiol (30 μg/kg/d); L-Arg = ovariectomized plus L-arginine (300 mg/kg/d); and Sham = sham surgery plus vehicle. Values are mean ± SEM. *$P < 0.05$ versus OVX; †$P < 0.05$ versus pretreatment; ††$P < 0.05$ versus pretreatment.

increasing concentration of endogenous ADMA in aged rats (15). It is possible that development of osteopenia after estrogen deficiency is related to increased ADMA concentration. In the study reported here, ovariectomy apparently led to osteopenia in rats, as indicated by the decrease of total BMD and BMD in L4–L6 and femurs and tibias, and a concomitant decrease in plasma NO values. However, differences in plasma concentration of ADMA were not observed in OVX rats or estrogen-treated rats. As mentioned previously, plasma ADMA concentration was increased in postmenopausal women; reasons for the discrepancy with our results are unclear. It is probable that the difference may be due to animal species differences.

Our recent work indicated that plasma ADMA concentration was increased in association with a decrease of BMD in aged rats (15). Similarly, increased ADMA concentration also has been seen in aged humans (5) suffering from developed osteoporosis. A number of studies have suggested that the increased ADMA concentration is related to an increase in oxidative stress (11, 22, 23). There is evidence that the degree of oxidative stress is increased in aged animals and humans (15, 16). However, in the study reported here, total BMD was decreased, but no changes in ADMA concentration have been documented in OVX rats. It has been reported that ovariectomy or estrogen replacement had no effect on the degree of oxidative stress (8). Those results support the hypothesis that oxidative stress may be an important factor contributing to ADMA production.

L-Arginine is the substrate of NO, and its administration promotes NO production, directly or indirectly (4, 14). We documented that L-arginine did not affect the plasma concentration of nitrite/nitrate in OVX rats. A similar effect has also been seen in healthy postmenopausal women and patients with coronary artery disease (2, 3). Others found that short-term treatment (six days) with L-arginine also had no effect on serum nitrite/nitrate values in OVX rats (29). The mechanism responsible for the lack of effect on NO synthesis by L-arginine is unclear. Possible explanation for these findings is that the cellular uptake of L-arginine or the availability of cofactor for endothelial NOS is limited.

Table 2. Effect of E2 or L-Arg on bone mineral density (g/cm^2) (n = 10/group)

Variable	Pre-treatment	Posttreatment			
	Total	Total	Spine	Femur	Tibia
OVX	0.1590 ± 0.0025	0.1628 ± 0.0017	0.1756 ± 0.0025	0.1806 ± 0.0028	0.1682 ± 0.0026
Sham	0.1651 ± 0.0031	0.1723 ± 0.0024**	0.2003 ± 0.0053**	0.1999 ± 0.0049**	0.1815 ± 0.0031**
E2 (L)	0.1620 ± 0.0032	0.1687 ± 0.0022	0.1907 ± 0.0043*	0.1966 ± 0.0031**	0.1780 ± 0.0026*
E2 (H)	0.1633 ± 0.0015	0.1712 ± 0.0028*	0.2016 ± 0.0050**	0.2001 ± 0.0043**	0.1816 ± 0.0026**
L-Arg	0.1614 ± 0.0037	0.1647 ± 0.0018	0.1761 ± 0.0028	0.1846 ± 0.0035	0.1716 ± 0.0025

*$P < 0.05$ versus OVX; **$P < 0.01$ versus OVX.
See Table 1 for key.

There is evidence that tetrahydrobiopterin (BH4), an important cofactor for eNOS, has been found to be significantly decreased in the aorta of OVX rats, compared with that in rats with intact ovaries, and exogenous BH4 significantly restored L-arginine-induced vasodilator responses in OVX rats (12, 14).

In conclusion, results of this study suggest that ovariectomy does not influence the plasma concentration of ADMA, and ADMA is not involved in ovariectomy-induced osteopenia in rats.

Acknowledgments

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