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# INFLUENCE OF ANDROSTENEDIONE ON SEDENTARY FEMALE RATS

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*ABSTRACT*

The purpose of this study was to examine the influence of androstenedione supplementation on plasma testosterone, cortisol, and cholesterol, mean arterial pressure (MAP), body weight, organ weights, and bone mineral density (BMD) in female rats. At age 10 weeks, an initial BMD measure was made. Rats in the andro group ($n$ = 13) received implants designed to deliver 1 μg androstenedione per gram body wt per day. Rats in the control group ($n$ = 13) received similar implants containing dextrose. After 8 weeks an arterial catheter was surgically implanted. MAP was measured for 30 minutes during home cage rest and a blood sample was taken for measurement of plasma cholesterol and testosterone. The next day animals were exposed to a novel stress (immersion in shoulder-deep, 37 °C water) for 15 minutes. MAP was measured before, during, and after the stress, and blood samples were taken for measurement of plasma cortisol. Animals were then euthanized and a final BMD measurement was made. The heart, liver, kidneys, adrenal glands, and ovaries were excised and weighed. Plasma cholesterol was significantly lower in the andro group, but there were no differences in MAP, plasma testosterone, plasma cortisol, body weights, organ weights, or BMD between groups.

† † †

Androstenedione is one of many intermediate compounds in steroid biosynthesis in the adrenal glands and the gonads. Cholesterol, the precursor for all steroid hormones, is converted first into 20 α-hydroxycholesterol by 20 α-hydroxylase and then into pregnenolone by desmolase. Pregnenolone can enter various biochemical pathways, resulting in the synthesis of mineralocorticoids (e.g., aldosterone), glucocorticoids (e.g., cortisol), androgens (e.g., testosterone), and estrogens (e.g., estradiol-17β). Testosterone may be metabolized in the liver to form 2α-, 16α-, and 6β-hydroxytestosterone, androstenedione, and 16α-hydroxyandrostenedione (Sugiyama et al. 1994), and both testosterone and androstenedione may be aromatized to estrogens or reduced to dihydrotestosterone in peripheral tissues (Longcope 1996). In females, androstenedione is produced by the theca calls of the corpus luteum, and under these circumstances it is rapidly converted to estrogen and/or progesterone. The production of androgens by theca cells appears to be regulated by insulin and insulin-like growth factors, explaining the association between hyperinsulinaemia and hyperandrogenism observed in women with polycystic ovarian syndrome (Lanzone et al. 1994, Nahum et al. 1995).

Examination of the influence of supplemental androstenedione on plasma levels of testosterone and changes in lean muscle mass and muscle strength have primarily focused on males. A single 100 mg dose of androstenedione failed to increase serum testosterone concentration in males, and long term (8–12 weeks) treatment with androstenedione did not enhance lean body mass or muscle strength in males participating in resistance training (King et al. 1999, Wallace et al. 1999). Ingestion of a single 100 mg dose of androstenedione in two healthy women, however, resulted in 4- and 7-fold increases in blood testosterone concentration (Mahesh and Greenblatt 1962), but information about long term treatment with androstenedione on lean body mass or muscle strength in females is lacking.

The adverse effects of long term anabolic steroid use (and abuse) are well known and include infertility, atrophy of the gonads, male pattern baldness in both sexes, decrease in body fat in females, short stature (when the steroid is taken during periods of growth), left ventricular hypertrophy, and liver tumors (National Institute on Drug Abuse Research Report Series, 2000). Long term treatment with androstenedione has been shown to have negative effects on the cardiovascular system. For example, serum high-density lipoprotein cholesterol levels in males were decreased following long term (8–12 weeks) treatment with androstenedione (King

et al. 1999). Previous work (Alen and Rahkila 1988, Alen et al. 1985, Costill et al. 1984, Haffner et al. 1983, Hurley et al. 1984, Kuipers et al. 1991, Peterson and Fahey 1984) suggests that elevated plasma levels of androgens are associated with an increased risk of cardiovascular disease in males. The influence of long term treatment with androstenedione on serum cholesterol levels in females is not known.

Cortisol stimulates gluconeogenesis by the liver, mobilizes amino acids from nonhepatic tissues, and mobilizes fatty acids from adipose tissues, enabling the body to resist stress. Previous studies have demonstrated an acute increase in plasma cortisol levels following exercise (Chandler et al. 1994, Hakkinen and Pakarinen 1993, Kraemer et al. 1993). The potential for supplemental androstenedione to enhance cortisol levels during stressful situations, and thus enable the body to cope more efficiency with the stressor, has not been investigated.

The increased bone turnover in postmenopausal women has been associated with decreased plasma levels of estrogen (Christiansen et al. 1982). There is evidence that plasma levels of androstenedione and testosterone are greater in premenopausal women compared with both peri- and postmenopausal women (Judd and Fournet 1994), and in postmenopausal women a positive relationship has been observed between oestradiol and testosterone levels and bone mineral density (Murphy et al. 1992). Moreover, androstenedione has been shown to protect against the development of osteopenia in ovariectomized rats, mediating its effect through androgen synthesis (Lea and Flanagan 1998). The potential role, however, of supplemental androstenedione in the development of peak bone density has not been investigated.

The purpose of this study was to examine the influence of long-term (10 weeks) treatment with androstenedione on plasma testosterone, cortisol, and cholesterol, mean arterial pressure (MAP), bone mineral density (BMD), body weight, and organ weights in female rats. Body weight was measured as an index of growth, and selected organs were weighed as indices of the potential influence of long term androstenedione treatment on the physiology of these organs. The hypotheses of this study were as follows: 1) long-term administration of the supplement androstenedione will have no influence on plasma levels of testosterone or cholesterol in sedentary female Long Evans rats at rest; 2) long-term administration of the supplement androstenedione will have no influence on plasma levels of cortisol in sedentary female Long Evans rats at rest or during exposure to novel immersion stress; 3) long-term administration of the supplement androstenedione will have no influence on the MAP of sedentary female Long Evans rats at rest or during exposure to novel immersion stress; 4) long-term administration of the supplement androstenedione will have no influence on the BMD of sedentary female Long Evans rats; 5) long-term administration of the supplement androstenedione will have no influence on the growth (as measured by increase in body weight) of sedentary female Long Evans rats; and 6) long-term administration of the supplement androstenedione will have no influence on the weight of the heart, liver, kidneys, adrenal glands, or ovaries of sedentary female Long Evans rats.

## METHODS

All methods were approved by the University of Nebraska at Kearney (UNK) Institutional Animal Care and Use Committee. Twenty-six female Long Evans rats (originally derived from Charles River stock) born between 06/04/99 and 06/12/99 in the UNK Copeland Hall animal facility were used in this study. At approximately 10 weeks of age, the rats were anesthetized with a mixture of ketamine (100 mg/kg i.p.; K-2753, Sigma Chemical Company, St. Louis, MO) and xylazine (20 mg/kg i.p.; X-1251, Sigma Chemical Company, St. Louis, MO) for measure of bone mineral density. Bone mineral density (BMD) was measured by dual energy x-ray absorptiometry (DPX-IQ®, LUNAR® Corporation, Madison, WI) using the forearm acquisition mode in the UNK Human Performance Laboratory. While the rats were still anesthetized, two, 1-cm pieces of Alliedsil® silicone tubing (I.D. 0.078"; Allied Biomedical, Paso Robles, CA) were implanted in the subcutaneous region between the scapulae. In half the rats (the androstenedione group, $n$ = 13), the silicone tubing contained 100 mg androstenedione plus filler (99.5% purity, ratio to filler 1:10; Ultimate Nutrition®, Inc., Farmington, CT) which delivered approximately 1 μg androstenedione per gram body weight per day for 8 weeks. The delivery rate was calculated based on the manufacturer's recommended daily dosage of androstenedione for adult humans. The ends of the silicone tubing were secured with a small piece of a wooden dowel and medical implant grade RTV adhesive (Applied Silicone Corp., Ventura, CA). The remaining rats (the control group, $n$ = 13) were implanted with two, 1 cm pieces of silicone tubing which contained an equal quantity of dextrose. The rats were maintained on normal Purina rat chow and water ad libitum and weighed weekly.

Eight weeks following implantation of the silicone tubing, animals were anesthetized as previously described and the left femoral artery was exposed. A catheter, constructed from a 6 cm piece of Teflon® tubing (I.D. 0.015"; Small Parts, Inc., Miami, FL) inserted into a 25 cm piece of Tygon® tubing (I.D. 0.02"; Cole-Parmer, Vernon Hills, IL), was placed in the femoral artery. The catheter was secured to the artery with 3-0 silk ties,

tunneled dorsally beneath the animal's skin, exited at the base of its neck, and secured with a surgical tether button (Harvard Apparatus, Holliston, MA). Following surgery, the animal was returned to its home cage, given normal rat chow and water ad libitum, and weighed daily. All animals were given 2 days to recover from surgery.

Following recovery from surgery, the animals remained in their home cages for a 30 minute measurement of resting mean arterial pressure (MAP) via a computerized data acquisition system (MacLab®, CB Sciences, Inc., Milford, MA). At the end of the 30 minute period, a 2.5 ml blood sample was slowly withdrawn from the arterial catheter and placed in a chilled microfuge tube containing 10 µl EDTA (90 mg/ml ethyleneglycol-bis[β-animoethyl ether] N,N,N",N"-tetraacetic acid, Sigma No. E-4378). The sample was centrifuged for 5 minutes at 2500 rpm, and the plasma was transferred to a clean microfuge tube and stored at -80 °C. The formed elements of the blood were resuspended in heparinized isotonic saline (67 USP units/ml) to a volume equaling 2.5 ml and returned to the animal.

The following day the animals were exposed to a novel, mild, physical stress, which consisted of being placed in a plastic 5-gallon bucket containing shoulder-deep 37 °C water for 15 minutes. MAP was measured as previously described for 15 minutes before the stress while the animal remained in its home cage, during the immersion stress, and for 15 minutes immediately after the stress when the animal was returned to its home cage. A 0.5 ml blood sample was taken before, immediately after, and 15 minutes after exposure to the immersion stress. The volume of the blood sample was immediately replaced with isotonic saline. The blood samples were treated as previously described, except that the formed elements of the blood were not returned to the animal.

The following day the animals were euthanized with an overdose of ether and the carcasses stored at -80 °C. Following collection of cardiovascular data and plasma samples from all animals, the carcasses were thawed and a second measure of BMD was made as previously described. In addition, the heart, liver, kidneys, adrenal glands, and ovaries were excised and weighed.

Plasma cholesterol was measured using an enzymatic, colorimetric assay (procedure 352, Sigma Diagnostics, St. Louis, MO). Plasma cortisol and testosterone were measured using radioimmunoassay (procedures 07-121002 and 07-189002, respectively, ICN Biochemicals, Inc., Costa Mesa, CA). All samples for each particular assay were processed simultaneously.

Bone mineral density was analyzed by highlighting the skull of the rat and selecting the "total" measure of BMD for the region. Cardiovascular data were analyzed by ANOVA with statistical significance ascribed for $p < 0.05$. Body weights, organ weights, BMD, and plasma cholesterol, cortisol, and testosterone were analyzed using a two-tailed, unpaired t-test with statistical significance ascribed for $p < 0.05$.

## RESULTS

There were no significant differences in body weight between the control group and the androstenedione group at any time during the study (Fig. 1). In addition, there were no significant differences in MAP between the control group and the androstenedione group during rest (Fig. 2) or during the stress session (Fig. 3). MAP was significantly elevated from rest within both groups during the immersion stress and throughout the recovery period, but there were no MAP differences within either group between the stress and recovery periods (Fig. 3). Plasma cortisol levels were not significantly different between groups during any of the sampling periods, although plasma cortisol was significantly elevated during the recovery period compared to the pre-stress period within the control group (Fig. 4).

There were no significant differences in organ weights or BMD between groups (Table 1), although BMD was significantly higher at the end of the study in both groups. Moreover, there was no significant difference in plasma testosterone at rest between groups (Table 1). Plasma cholesterol (total), however, was significantly lower in animals in the androstenedione group compared to animals in the control group (Table 1).

## DISCUSSION

The major limitation of this study was that circulating levels of androstenedione were not evaluated, so any conclusions about the potential influence of androstenedione on the parameters in question are suspect. Plasma androstenedione can be measured using radioimmunoassay, but, unlike the radioimmunoassay procedures for measurement of testosterone and cortisol which utilize $^{3}H$, the procedure for measurement of androstenedione utilizes $^{125}I$. Data concerning circulating levels of androstenedione would, of course, permit conclusions to be based on the potential influence of androstenedione. The primary investigator's institution is not licensed to use this isotope, so evaluation of circulating levels of androstenedione was not possible. It is entirely possible that the implants became encased with subcutaneous fat or, for some other reason, failed to deliver androstenedione at the desired dosage (or at all). Moreover, since the animals were not ovariectomized, it is also possible that any increase in plasma androstenedione was offset by a decrease in ovarian activity.

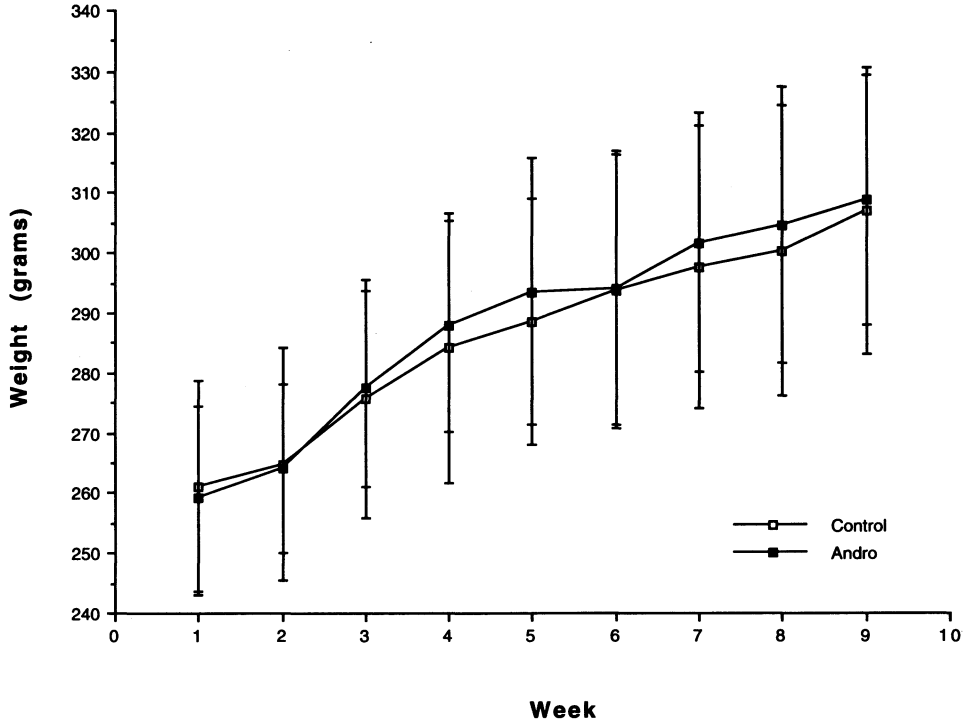


Figure 1. Weekly body weights of female Long Evans rats. Rats in the andro group ($n$ = 13) were treated with androstenedione (surgical implant to deliver 1 μg androstenedione per gram body weight daily for 8 weeks) whereas animals in the control group ($n$ = 13) were treated with dextrose. Week 1 represents weight on day implant was surgically put in place. Values expressed as mean ± standard deviation.

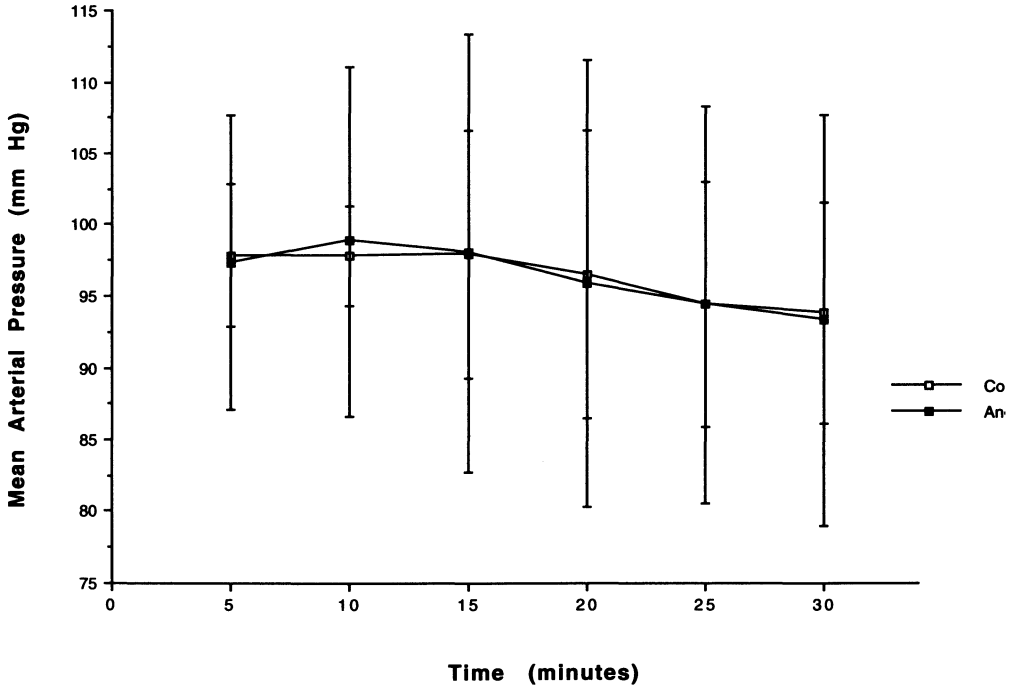


Figure 2. Mean arterial pressure of female Long Evans rats during a 30 minute home cage rest session. Rats in the andro group ($n$ = 13) were treated with androstenedione (surgical implant to deliver 1 μg androstenedione per gram body weight daily for 8 weeks) whereas animals in the control group ($n$ = 10) were treated with dextrose. Values expressed as mean ± standard deviation.

Therefore, plasma androstenedione levels may have not been different between groups.

The only significant difference observed between groups was the lower level of total plasma cholesterol in the androstenedione group. This is consistent with the general observations of other investigators (King et al. 1999), who demonstrated that long term treatment with androstenedione lowered serum high-density lipoprotein cholesterol levels. Again, due to limitations in the resources available to the primary investigator, only total plasma cholesterol and not the lipoprotein profile of these animals was measured in this study. If the lower total plasma cholesterol level reflects a decrease in low-density or very-low density lipoprotein cholesterol levels, this would suggest that the treatment was beneficial to the cardiovascular system. If the lower total plasma cholesterol level reflects a decrease in high-density lipoprotein cholesterol levels, however, then the treatment would be considered to be potentially deleterious to the cardiovascular system.

The results of this study demonstrated no influence on the cardiovascular activity in sedentary female Long Evans rats at rest or during exposure to novel stress. While testosterone may have a direct role in the increased risk of cardiovascular disease observed in males (Adams et al. 1995, Bruck at al. 1997), short-term administration of testosterone has been shown to have relaxing effects on coronary arteries in both animals (Chou et al. 1996, Yue et al. 1995) and humans (Webb et al. 1999). Again due to the limitations of the study, it is not possible to conclude that long-term administration of androstenedione influences cardiovascular activity.

There were no differences in plasma levels of testosterone and cortisol between groups in this study. King et al. (1999) and Wallace et al. (1999) reported no increases in plasma levels of testosterone in response to acute ingestion of androstenedione. While significant increases in serum estradiol and estrone levels have been observed following long-term (8 weeks) androstenedione supplementation (King et al. 1999), no increases in serum testosterone were observed. While no

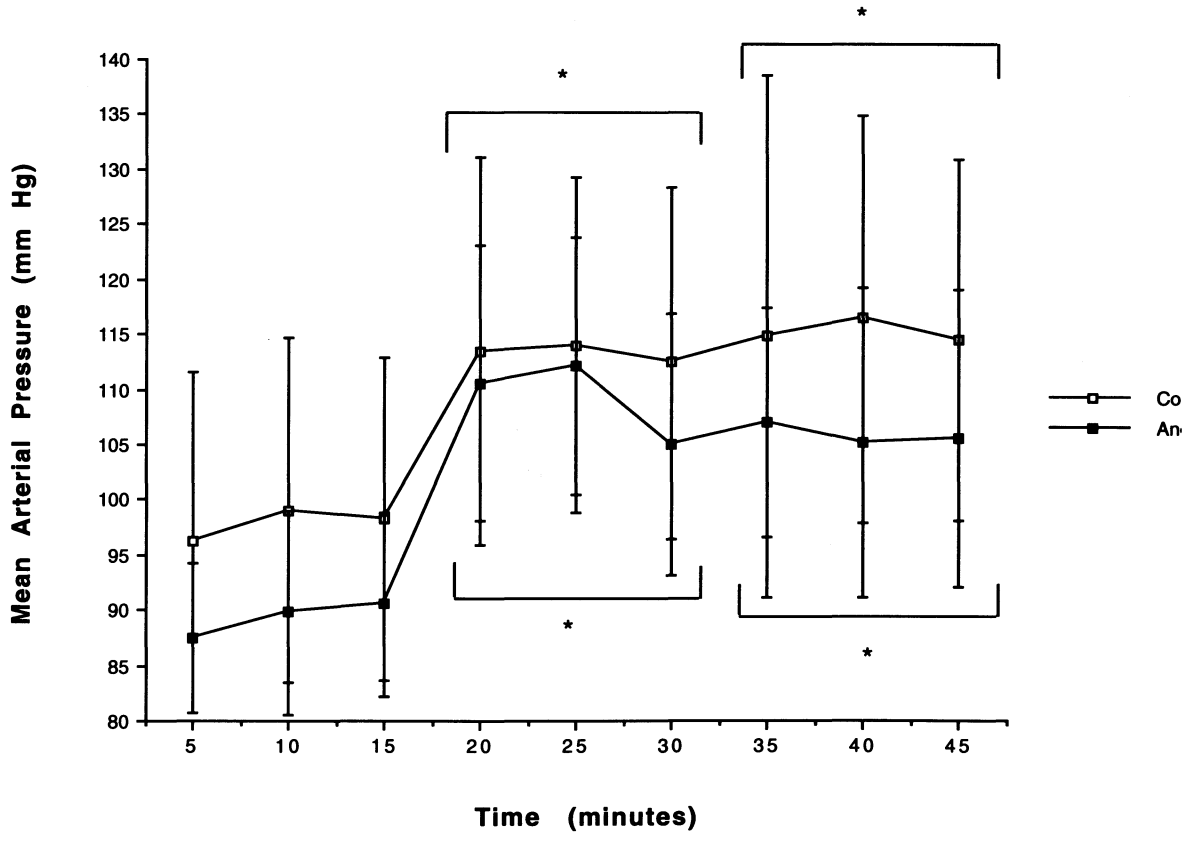


Figure 3. Mean arterial pressure of female Long Evans rats during a novel stress (15-minutes immersion in shoulder-deep 37°C water) session. Rats in the andro group ($n$ = 13) were treated with androstenedione (surgical implant to deliver 1 μg androstenedione per gram body weight daily for 8 weeks) whereas animals in the control group ($n$ = 10) were treated with dextrose. Minutes 5–15 represent a 15 minute home cage pre-stress period, minutes 20–30 represent the 15 minute stress period, and minutes 35–45 represent a 15 minute home cage recovery period. Values expressed as mean ± standard deviation. *Significantly different from pre-stress period within group, $p < 0.05$.

previous work has been done examining the influence of long-term treatment with androstenedione on plasma cortisol levels, the limitations of the present study do not permit any conclusions on the influence of androstenedione supplementation on plasma cortisol levels.

There were no differences in body weight between groups in this study. While this observation is relatively consistent with the results of other investigators (King et al. 1999, Wallace et al. 1999) who reported that long term androstenedione supplementation failed to enhance lean body mass in males, the limitations of the study do not permit any conclusions about the influence of androstenedione on either body composition or growth in female subjects. In addition, there were no differences in organ weights between groups. Long term anabolic steroid use is associated with hypertrophy of the heart and atrophy of the gonads (National Institute on Drug Abuse Research Report Series, 2000), and the liver and kidneys must metabolize and excrete waste products, respectively, from supplemental steroids. Again, due to the limitations of this study, no conclusions about the influence of supplemental androstenedione on organ function are possible.

Bone mineral density was significantly greater in both groups at the conclusion of the study compared with the beginning of the study, but the animals were only 10 weeks of age at the beginning of the study, so this increase in BMD is consistent with the normal changes in BMD associated with growth. While previous work suggests that supplemental androstenedione can protect against osteopenia (Lea and Flanagan 1998) and that postmenopausal women with higher androgen levels have greater bone mass (Murphy et al. 1992), the results of this study do not indicate any influence on peak bone mass development.

In summary, the inability to measure circulating levels of androstenedione in both groups of animals severely impacts the ability to make any conclusions concerning the influence of long term androstenedione treatment on plasma testosterone, cortisol, and cholesterol, MAP, BMD, body weight, and organ weights in

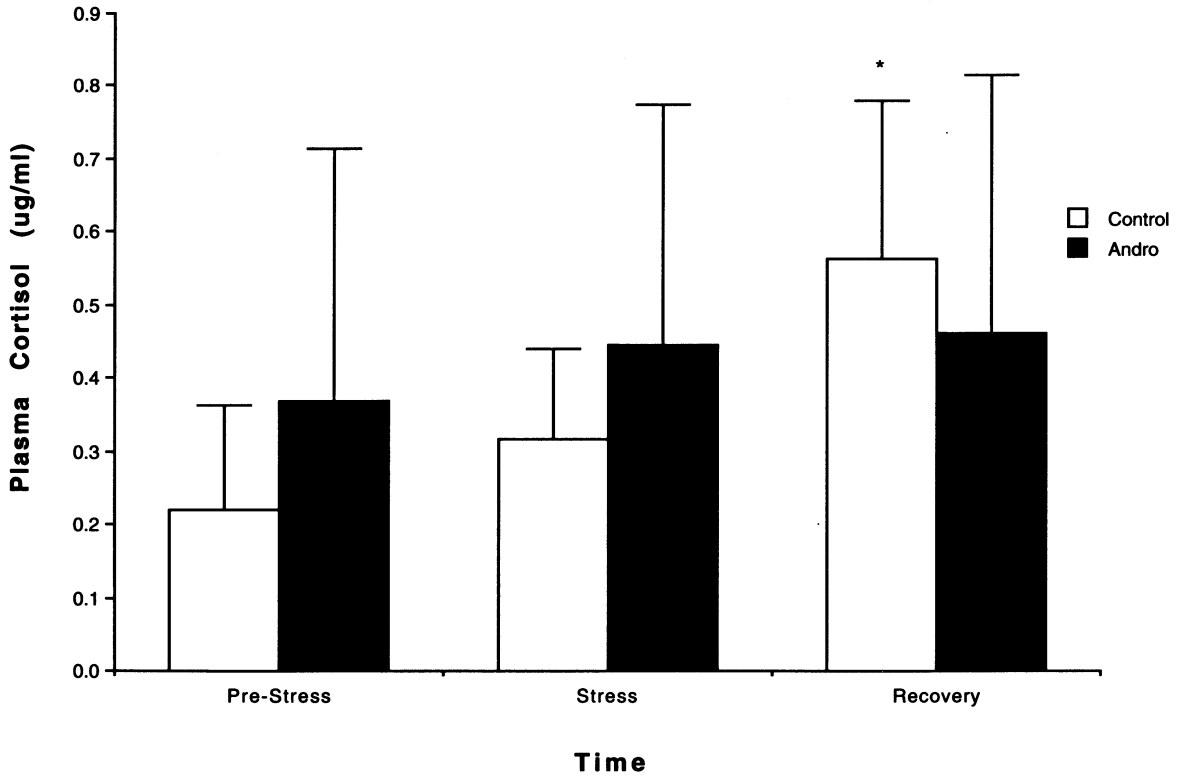


Figure 4. Plasma cortisol levels of female Long Evans rats exposed to a novel stress (15-minutes immersion in shoulder-deep 37°C water) session. Rats in the andro group ($n$ = 13) were treated with androstenedione (surgical implant to deliver 1 μg androstenedione per gram body weight daily for 8 weeks) whereas animals in the control group ($n$ = 10) were treated with dextrose. Plasma samples were taken following a 15 minute home cage pre-stress period, following the 15 minute stress period, and following a 15 minute home cage recovery period. Values expressed as mean ± standard deviation. *Significantly different from pre-stress, $p < 0.05$.

Table 1. Body weight at time of surgery, organ weights, bone mineral density (BMD), and resting levels of plasma testosterone and cholesterol of female Long Evans rats. Rats in the androstenedione group ($n$ = 13) were treated with androstenedione (surgical implant to deliver 1 μg androstenedione per gram body weight daily for 8 weeks) whereas animals in the control group ($n$ = 13 except for plasma data where $n$ = 10) were treated with dextrose. Values are expressed as mean ± standard deviation.

| | Androstenedione group | Control group |
|---|---|---|
| Body weight (g) at surgery | 315.9 ± 24.9 | 309.8 ± 21.0 |
| Heart (whole) (mg) | 871 ± 80 | 879 ± 88 |
| Liver (g) | 11.978 ± 0.686 | 11.763 ± 1.777 |
| Kidneys (g) | 2.339 ± 0.172 | 2.352 ± 0.297 |
| Adrenal glands (mg) | 92 ± 13 | 83 ± 14 |
| Ovaries (mg) | 85 ± 20 | 87 ± 19 |
| BMD (initial) (g/cm$^2$) | 0.314 ± 0.032 | 0.315 ± 0.025 |
| BMD (final) (g/cm$^2$) | 0.378 ± 0.035# | 0.364 ± 0.031# |
| Plasma testosterone (ng/ml) | 0.0851 ± 0.0150 | 0.0836 ± 0.0085 |
| Plasma cholesterol (total; mg/dl) | 78.1 ± 52.4 | 141.9 ± 89.5* |

* Statistically different from androstenedione group, $p < 0.05$.
\# Statistically different from initial BMD measure, $p < 0.05$.

female rats. Given that most of the information concerning the effects of long term androstenedione treatment focus on the influence of this supplement on male subjects, further investigation of the effects of androstenedione on female subjects is warranted.

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