Effects of hyperandrogenemia and increased adiposity on reproductive and metabolic parameters in young adult female monkeys


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Submitted 6 June 2013; accepted in final form 12 April 2014

McGee WK, Bishop CV, Pohl CR, Chang RJ, Marshall JC, Pau FK, Stouffer RL, Cameron JL. Effects of hyperandrogenemia and increased adiposity on reproductive and metabolic parameters in young adult female monkeys. Am J Physiol Endocrinol Metab 306: E1292–E1304, 2014. First published April 15, 2014; doi:10.1152/ajpendo.00310.2013.—Many patients with hyperandrogenemia are overweight or obese, which exacerbates morbidities associated with polycystic ovary syndrome (PCOS). To examine the ability of testosterone (T) to generate PCOS-like symptoms, monkeys received T or cholesterol (control) implants (n = 6/group) beginning prepubertally. As previously reported, T-treated animals had increased neuroendocrine drive to the reproductive axis [increased luteinizing hormone (LH) pulse frequency] at 5 yr, without remarkable changes in ovarian or metabolic features. To examine the combined effects of T and obesity, at 5.5 yr (human equivalent age: 17 yr), monkeys were placed on a high-calorie, high-fat diet typical of Western cultures [Western style diet (WSD)], which increased body fat from <2% (pre-WSD) to 15–19% (14 mo WSD). By 6 mo on WSD, LH pulse frequency in the controls increased to that of T-treated animals, whereas LH pulse amplitude decreased in both groups and remained low. The numbers of antral follicles present during the early follicular phase increased in both groups on the WSD, but maximal follicular size decreased by 50%. During the late follicular phase, T-treated females had greater numbers of small antral follicles than controls. T-treated monkeys also had lower progesterone during the luteal phase of the menstrual cycle. Although fasting insulin did not vary between groups, T-treated animals had decreased insulin sensitivity after 1 yr on WSD. Thus, while WSD consumption alone led to some features characteristic of PCOS, T caused a more severe phenotype with regard to insulin sensitivity, increased numbers of antral follicles at midcycle, and decreased circulating luteal phase progesterone levels.

testosterone; obesity; amenorrhea; insulin insensitivity; ovarian follicle

POLYCYSTIC OVARY SYNDROME (PCOS) affects 4–8% of reproductive-aged women (5, 48) and is characterized by hyperandrogenemia (HA), irregular menstrual cycles, and polycystic ovaries, in addition to rapid luteinizing hormone (LH) pulse frequency, increased LH response to gonadotropin-releasing hormone (GnRH), decreased sensitivity to progesterone (P4), negative feedback, and decreased insulin sensitivity (SI) (13, 20, 90). Moreover, up to 90% of women with PCOS are overweight or obese, which is higher than the general population of the United States, where 65% of women over the age of 20 are overweight or obese (6, 33). Women with PCOS also tend to have a central distribution of adiposity, which is associated with increased rates of high blood pressure and cardiovascular disease (23, 51).

Being overweight or obese aggravates many symptoms of PCOS. Basal production of testosterone (T) and free T are reportedly elevated in obese, compared with nonobese, PCOS patients (2, 39, 45, 63). Hirsutism, a clinical marker of elevated androgens, is also more severe in obese PCOS women (45). Obese PCOS patients also have higher rates of oligo- and amenorrhea, infertility, and miscarriage compared with lean women with the disorder (8, 45, 65). Additionally, between 50 and 70% of women with PCOS suffer from some degree of insulin insensitivity, and SI scores worsen with increasing body mass index (BMI) (21, 28, 43, 62). Compensatory hyperinsulinemia follows a similar pattern, with obese PCOS women having the highest insulin secretion (20). Many PCOS patients resume menstruation and ovulation with even mild weight loss, although this strategy is not always successful at normalizing reproductive function (48, 67).

Obesity in peripubertal girls is also associated with HA (57), and this is thought to be a forerunner of adult PCOS (34, 88). Like adults with PCOS, obese girls with HA have increased levels of LH and decreased sensitivity to P4 negative feedback (22, 49). In late puberty, girls with obesity reportedly have increased frequency of pulsatile LH release compared with nonobese girls (57). In addition, obese girls are more likely to have irregular menstrual cycles during late puberty compared with nonobese controls (57). As with adults, hyperinsulinemia is also more common in adolescents with PCOS, especially in those who are obese (76).

Cumulatively, there is substantial evidence that PCOS is characterized by both HA and obesity. However, there are women with PCOS who are normal weight (6, 53), indicating that, while obesity likely aggravates symptoms of PCOS, it is probably not the sole cause of PCOS. In a previous study of female monkeys that were treated chronically with T beginning at 1 yr of age, we observed that elevated serum T levels led to
an increased central drive to the reproductive axis (59), similar to what is seen in women with PCOS (3, 7, 86). However, there were no robust group differences in ovarian or metabolic function after 4 yr of continuous exposure to slightly elevated T. Thus, based on evidence that obesity aggravates PCOS symptoms in women, it was of interest to investigate whether weight gain would also lead to the development of ovarian or metabolic symptoms in these T-treated monkeys. All females from the original cohort (59) were placed on a high-calorie, high-fat Western style diet (WSD). We hypothesized that there would be a continued effect of T on neuroendocrine function, in addition to the development of an ovarian and metabolic PCOS-like phenotype in the T-treated animals.

MATERIALS AND METHODS

Animals

Twelve female rhesus macaques (Macaca mulatta) that were 5.5 yr of age (young adults) at the beginning of this study were used for this protocol and were the same animals that we reported on previously (59). All monkeys were housed individually in single cages (81 × 61 × 69 cm) in a temperature-controlled room (24 ± 2°C), with lights on for 12 h/day (0700–1900). Monkeys were trained to approach the front of their cage so menses could be detected daily throughout the study by vaginal swabbing. The first day of menses was designated day 1 of a menstrual cycle. All procedures in this study were reviewed and approved by the Oregon National Primate Research Center (ONPRC) Institutional Animal Care and Use Committee.

Western Style Diet

Until the monkeys were 5.5 yr of age, they were fed two meals of Purina LabDiet fiber-balanced monkey chow each day (no. 5000; Purina Mills, St. Louis, MO), supplemented with fresh fruits and vegetables. Thereafter, their diet was switched from fiber-balanced monkey chow (15% calories from fat, 21% from protein, 59% from carbohydrates) to a diet that was specifically formulated to mimic a typical WSD (33% calories from fat, 17% from protein, 51% from carbohydrates; 5A1F; Purina Mills) (80, 81). The animals were fed ad libitum, and each meal was supplemented with a high-sugar, high-calorie treat (e.g., a cookie, snack cake, etc.). Previous studies have shown that monkeys eating this diet gained over 10% of their body weight within the first 5–6 mo of being on this diet (80, 82).

Testosterone and Cholesterol Implants

T and cholesterol implants were prepared and placed subcutaneously as described previously (59). Blood samples were taken weekly to ensure that the implants sustained a three- to fourfold increase in T. Thus, based on evidence that obesity aggravates PCOS symptoms in women, it was of interest to investigate whether weight gain would also lead to the development of ovarian or metabolic symptoms in these T-treated monkeys. All females from the original cohort (59) were placed on a high-calorie, high-fat Western style diet (WSD). We hypothesized that there would be a continued effect of T on neuroendocrine function, in addition to the development of an ovarian and metabolic PCOS-like phenotype in the T-treated animals.

Blood Collection

Blood samples were collected weekly from all animals to track serum P₄, estradiol (E₂), and T concentrations. Small aliquots of blood were collected through the remote sampling system and processed as described previously (59). Plasma was removed, and the red blood cells were sterilely resuspended in saline and reinfused through the catheter system to the animal. Each week’s samples were assayed for T, and, when an individual animal’s serum T concentration fell below the threshold of 1.2 ng/ml, the implant was replaced. Cholesterol implants were also changed regularly so that the control animals received the same average number of implant surgeries as the T-treated animals. Blood samples were also collected daily during one menstrual cycle in each animal (at 6.5 yr of age) to track the patterns and levels of E₂ and P₄ during the follicular and luteal phases. More frequent samples were collected during the neuroendocrine and glucose tolerance testing (GTT) protocols, as reported previously (59).

Assays

T was measured by ELISA (catalog no. IB79106; IBL America, Minneapolis, MN) by the Endocrine Technology Support Core (ETSC) Laboratory at the ONPRC. The sensitivity of the T assay was 0.08 ng/ml, and the intra- and interassay coefficients of variation for the assays were 8.9 and 13.5%, respectively. Both E₂ and P₄ were also assayed by the ETSC Laboratory using the Immulite 2000 platform as previously described (59). As with many validated clinical platforms, the Immulite 2000 runs three quality control (QC) serum pools daily, and, as such, no specific intra-assay QC data are available. The interassay coefficient of variation, reflecting variability in daily QC results over the period in which these assays were performed was 8.5% for E₂ and 9.4% for P₄. Sex hormone-binding globulin (SHBG) was measured by a monkey-specific SHBG ELISA validated by the ETSC (catalog no. 251544; Hözel Diagnostika, Kiel, Germany). The sensitivity of the SHBG assay was 9.6 pmol/ml, and the intra-assay coefficient of variation was 4.9%.

Physical Activity

Physical activity levels were assessed continuously, as described previously (41), for the final 10 mo of this study, when the animals were 6–7 yr of age. The animals were on the WSD for the entire duration that activity was monitored. Animals wore jackets that contained a pocket housing a three-way accelerometer (Actical; Respironics, Bend, OR). The monitors were programmed to record total activity counts per minute, and data were downloaded approximately...
one time every 3 wk when monkeys were sedated with ketamine hydrochloride.

Experimental Protocols

Reproductive and metabolic parameters were measured before (pre-WSD) and after (up to 16 mo) onset of the WSD (see Fig. 1). The animals began WSD consumption at 5.5 yr of age.

Experiment 1. Evaluation of neuroendocrine function of the reproductive axis. Pulsatile LH secretion was measured during the early follicular phase (D1–3) of the menstrual cycle in the animals when they were 5 yr (pre-WSD, data previously reported in Ref. 59), and again after 6 and 14 mo on the WSD. Small blood samples (0.4 ml/sample) were collected as described previously (59) every 10 min from 1300 to 0100. The early follicular phase was chosen because this is the time period when differences in pulsatile LH secretion are most apparent between women with PCOS and healthy women (56), and when differences were seen between the groups in our previous studies (59).

Pulsatile LH secretion was also measured during the luteal phase of a menstrual cycle after 6 and 14 mo on the WSD. Daily blood samples were taken beginning on D8 of the menstrual cycle and were assayed to track P4 levels on a day-by-day basis. On the first day that P4 levels were ≥2.0 ng/ml, pulsatile LH secretion was measured using the methods described above. A threshold of 2.0 ng/ml was chosen because it indicates a functional corpus luteum and is a standardized functional level of P4 (i.e., prevents menstruation).

LH responsiveness to GnRH (National Hormone and Peptide Program, Harbor-UCLA Medical Center) was measured between 0900 and 1000 on D2–4 of a menstrual cycle at 5 yr of age (pre-WSD) and after 6 and 14 mo on the WSD, as previously described (59). GnRH (250 ng/kg iv) was infused at time (t) 0 and blood samples (0.4 ml/sample) were collected at −15, −8, −1, 15, 30, 60, and 90 min (19, 59). This dose was chosen so that monkeys would receive a physiological dose that caused an LH response, but a response that was submaximal to allow detection of individual differences in LH responsiveness.

Experiment 2. Ovarian structure-function. Weekly (throughout the protocol) and daily (for one menstrual cycle, 14 mo after beginning WSD) blood samples were assayed for E2 and P4 as described above, and the presence of the follicular and luteal phases of the menstrual cycle were determined based on these data. From the daily samples, the length of the follicular phase was denoted as the number of days from onset of menses to the day of the peak E2 value (inclusive). The luteal phase length was defined as occurring from the day after the peak E2 value to the day before onset of the next menses (inclusive). Ovarian ultrasounds were performed on D1–3 of a menstrual cycle when the animals were 5 yr old (pre-WSD, data reported previously, see Ref. 59) and 3 and 14 mo after onset of WSD. At 14 mo WSD, ultrasounds were also performed during the late follicular phase, once E2 levels reached >100 pg/ml or at 10 days postmense, whichever occurred first. A GE Medical Systems Voluson 730 Expert Doppler ultrasound instrument (GE Healthcare, Waukesha, WI) with both two-dimensional (2D, 4.5–16.5 MHz) and four-dimensional (4D, 3.3–9.1 MHz) transabdominal probes was used, as described by Bishop et al. (12). Animals were assigned random identifiers so the ultrasonographer (Bishop) remained blind to the treatment group. The 2D probe was used to orient the image field to the uterus and identify the ovaries. The 4D probe was then used to generate a data file of each individual ovary, which included a series of images collected in one scan through the entire ovary. Ovaries were analyzed for circumference, number of antral follicles (0.5 mm diameter or greater) in each ovary, and the mean, maximum, and minimum size of the antral follicles in each ovary, using previously defined methods for adult female rhesus monkeys (12), and as reported previously (59). All parameters were then decoded for comparisons between treatment groups.

Experiment 3. Metabolic parameters. GTT was performed during the early follicular phase of a menstrual cycle when the animals were 3.5 yr of age (pre-WSD; data reported previously in Ref. 59) and 12 mo after onset of WSD. For monkeys not showing regular menstrual cycles, the GTT was performed when a blood sample indicated that E2 and P4 levels were low, and thus the absence of a dominant follicle or corpus luteum in the ovaries. Each animal was sedated initially with telazol (tiletamine hydrochloride and zolazepam hydrochloride, 2–6 mg/kg im; Fort Dodge Animal Health, Fort Dodge, IA) and subsequently with ketamine to maintain sedation. The protocol was based on that designed by Bergman et al. (11) and was described previously (59). Dextrose (300 mg/kg) was infused intravenously through the catheter system, and blood samples were taken from 15 min before to 3 h after the glucose infusion. Tolbutamide (5 mg/kg) was infused intravenously 20 min after the dextrose to stimulate the pancreas to secrete more insulin. All samples were immediately assayed for glucose using the YSI 2300 Stat Plus (YSI, Yellow Springs, OH) and subsequently for insulin by RIA (Linco Human Insulin RIA; Millipore, Billerica, MA). The sensitivity of the insulin assay was 1 μIU/ml, and the intra-assay coefficient of variation was 4.9%.

Metabolic rate was measured over a 24-h period when the animals were 4.5 yr old (pre-WSD) and 12 mo after onset of WSD, using previously published methods (81, 82). The animals were placed in a sealed Lexan and stainless steel metabolic chamber (Columbus Instruments, Columbus, OH) at −1000. Fresh air was pumped in and circulated with a 4-in. fan. The amounts of oxygen consumed and carbon dioxide produced were measured using a computer-controlled open-circuit calorimeter, and total energy expenditure (kcal) was calculated using the Oxymax system (Columbus Instruments). The animals did not receive their normal meals during this time but were fed a 110-g banana at 1500.

Dual-energy X-ray absorptiometry (DEXA) scanning was performed at 5 yr of age (pre-WSD; data reported previously, see Ref. 59) and 16 mo after onset of WSD. Percent body fat, percent central fat, fat mass, and lean tissue mass were determined using DEXA scanning. Monkeys were sedated with ketamine and positioned supine on the bed of a Hologic Dexascan scanner (Discovery scanner; Hologic, Bedford, MA). Two to three scans were performed for each monkey in “infant whole body” mode, and averages were calculated for each measure. To delineate central fat mass from peripheral fat mass, fat in the trunk (including both the subcutaneous and visceral compartments) and fat in the extremities were calculated using standard methodology (24, 59).

Statistical Analysis

Pulsatile LH secretion. LH pulses were identified using the Pulsar algorithm developed by Merriam and Wachter (62), and used previously to detect LH pulses in these monkeys (59). Pulse frequency was defined as the number of Pulsar-detected pulses in 12 h and the
following G values were used: G(1, 50.00); G(2), 1.0; G(3), 0.40; G(4), 0.40; and G(5), 0.40. Pulse amplitude was calculated by subtracting the baseline LH level from the peak LH level of each pulse. For all analyses, LH values below the level of detectability for the assay were assigned the minimum detectable concentration of the assay. Independent Student’s t-tests were used to determine group differences in the number of pulses, proportion of daytime (vs. nighttime) pulses, pulse amplitude, and LH response to GnRH. For the GnRH stimulation, LH area under the curve (AUC) was calculated, correcting for baseline LH levels.

Ovarian morphology, function, and interactions. Changes in total numbers of antral follicles measured by ultrasound analysis on D1–3 (combining counts from both ovaries in each animal) from before and throughout WSD were analyzed by the mixed-models procedure (treatment × time). Numbers of antral follicles present on each ovary during the late follicular phase (after possible selection of the dominant follicle) and P_4 AUC levels during the luteal phase were analyzed by ANOVA (main effect of treatment). Post hoc tests employed were paired t-tests with a Bonferroni correction for multiple comparisons. Pearson r correlations were used to examine relationships between neuroendocrine, antral follicle counts (by ovary), and metabolic parameters independently of T exposure to determine the main effect of WSD on ovarian structure-function.

Metabolic parameters. The MINMOD Millennium computer program was used to determine glucose effectiveness (Sg), SI, acute insulin response to glucose (AIRg), and disposition index (DI) values (15). This program was designed to calculate these values based on the GTT protocol that was described by Bergman and colleagues (11) and used in this study. Sg indicates the capacity of glucose to mediate its own disposal, AIRg addresses the adequacy of insulin secretion, and SI quantifies the capacity of insulin to promote glucose disposal. DI is the product of AIRg and SI, and it therefore takes into account insulin concentration and action (10, 15). Independent-sample t-tests were used to determine group differences in GTT measures.

Basal metabolic rate was calculated as the average number of kilocalories expended per kilogram per hour from 2300 to 0300. This time period is when monkeys are typically asleep and their heart rate is slowest (Cameron, unpublished data). BMI (kg/m²) was calculated using the crown-rump length as height. The free androgen index was calculated as T/SHBG × 100. An independent t-test was used to assess group differences in basal metabolic rate and body composition parameters.

Mixed-measures ANOVAs were used to determine changes in all measures across time. Some data from this study were compared with data collected from the same animals before WSD treatment (59), when the monkeys were between 3.5 and 5 yr of age. Statistical analyses of ovarian function and morphology were performed using SAS (version 9.2; SAS Institute, Cary, NC). All other analyses were performed using IBM SPSS Statistics 20 (SPSS, Chicago, IL). All values are presented as means ± SE. Significance was set at P < 0.05.

RESULTS

Weight Increase and Testosterone Concentrations

The animals increased their daily caloric intake by an average of 140 ± 13% when switched from normal chow to the WSD (before WSD: 467 ± 0 kcal/day; after WSD: 1,121 ± 62 kcal/day), but there were no differences between T-treated and control animals with regard to average kilocalories of WSD consumed per day (P = 0.95). All animals increased their body weight by at least 10% while on the WSD (average percent gain for T-treated animals: 29.4 ± 7.5%, controls: 27.3 ± 4.0%). When weight gain over the entire postpubertal period was analyzed (i.e., from 3.5 to 7 yr of age), there was a significant effect of treatment [F(1,10) = 5.19, P = 0.046], with T-treated animals gaining more weight than controls, although percent weight gain on the WSD was not different between the groups (P > 0.1). Weight gain while on the WSD was positively correlated with caloric intake (r = 0.78, P = 0.003). BMI increased from 21.8 ± 0.5 kg/m² before the WSD to 25.6 ± 1.2 kg/m² after 16 mo on the WSD (P = 0.002), and was not different between the groups (P > 0.1). There was a strong trend toward T-treated animals being less active than controls over the 10-mo period that activity was measured [average activity counts/day: T treated, 201,852 ± 54,929; control, 423,053 ± 84,067; t(10) = −2.20, P = 0.052]. However, there was no correlation between weight gain during WSD consumption and average daily activity during the last 10 mo of the study (r = −0.17, P = 0.60). There was also no correlation between postpubertal weight gain and average daily activity during the final 10 mo of the study (r = −0.001, P = 0.997).

Plasma T concentrations in T-treated animals were maintained in a narrow range regardless of weight gain; levels were 4.2 ± 0.2-fold higher than in control animals from the time of first implant at 1 yr of age through 7 yr of age (T treated, 1.71 ± 0.05 ng/ml; control, 0.46 ± 0.02 ng/ml; P = 0.001). SHBG levels did not change from before to 16 mo after onset of WSD, and were not different between the groups either before or after the WSD (before WSD: T treated, 13.1 ± 1.1 pmol/ml; control, 13.1 ± 1.0 pmol/ml; after WSD: T treated, 14.4 ± 1.5 pmol/ml; control, 12.8 ± 1.1 pmol/ml; P > 0.1). The free androgen index was higher in T-treated animals compared with controls both before and after WSD consumption (both P < 0.01), and decreased slightly after consuming the WSD in both groups (before WSD: T treated, 11.6 ± 1.6; control, 2.3 ± 0.4; after WSD: T treated, 9.3 ± 1.9; control, 1.1 ± 0.1). Implants for both groups were replaced an average of every 9.2 ± 0.4 wk throughout the study.

Experiment 1. Neuroendocrine Function of the Reproductive Axis

When assessing the change in LH pulse frequency from before to 14 mo after starting the WSD, there was a significant diet × treatment interaction [F(1,9) = 8.8, P = 0.016]. This resulted from an increase in pulse frequency in the control but not the T-treated animals from before to 6 mo after beginning the WSD [t(5) = −5.0, P = 0.004; Fig. 2A]. LH pulse number did not change in either group when comparing the data from 6 mo with 14 mo on WSD [before WSD: T treated, 9.7 ± 1.8 pulses/12 h; control, 3.7 ± 1.8; after 6 mo WSD: T treated, 11.4 ± 1.0; control, 13.3 ± 1.1; after 14 mo WSD: T treated, 13.0 ± 1.0; control, 12.3 ± 1.0; Fig. 2A]. There was a significant effect of diet on LH pulse amplitude, which decreased in both groups by 6 mo after the animals switched to the WSD and remained lower after 14 mo [before WSD: T treated, 0.73 ± 0.21 ng/ml; control, 0.70 ± 0.15 ng/ml; after 6 mo WSD: T treated, 0.16 ± 0.02 ng/ml; control, 0.38 ± 0.12 ng/ml; after 14 mo WSD: T treated, 0.27 ± 0.04 ng/ml; control, 0.34 ± 0.11 ng/ml; F(1,8) = 13.1, P = 0.007; Fig. 2B]. There was no effect of time or treatment, nor was there a time × treatment interaction on LH response to exogenous GnRH (either assessed as LH AUC or peak LH, data not shown; all P > 0.1).
Six months after starting the WSD, the effect of P₄ negative feedback on pulsatile LH secretion was examined by comparing LH pulsatility measured during the early follicular phase (D1–3) with that measured during the luteal phase on the day that plasma P₄ was first over 2 ng/ml (Fig. 3, top). Two control animals and one T-treated animal were not tested because of either lack of ovulation or catheter removal. The analyses were performed on the remaining four controls and five T-treated monkeys. There were no group differences in LH pulse frequency, LH pulse amplitude, or P₄ level measured during the luteal phase pulse bleeds (all P > 0.1). Most animals showed a decrease in LH pulse frequency measured during the luteal phase compared with the values on D1–3, and there were no group differences in percent suppression \( t(7) = -0.88, P = 0.4; \) Fig. 3). This experiment was performed again after 14 mo on the WSD (Fig. 3, bottom). Two T-treated animals were not tested due to catheter removal, so analyses were performed on the remaining four T-treated and six control animals. Similar to the results after 6 mo on the WSD, most animals showed a decrease in pulse frequency during the luteal phase compared with the D1–3 data. Again, however, there were no group differences in LH pulse frequency or amplitude, or percent suppression between the follicular and luteal measures (all P > 0.1; Fig. 3). When analyzing data from the four animals from each group that were tested after both six and 14 mo of WSD, there was a weak effect of time on percent suppression of LH during the luteal phase [6 mo WSD: 29.6 ± 9.5% suppressed; 14 mo WSD: 42.0 ± 9.0% suppressed; \( F(1,3) = 6.16, P = 0.09 \)], but there was no effect of T treatment (P > 0.1).

**Experiment 2. Ovarian Structure-Function**

When comparing the year before with the year after beginning the WSD, animals had an increased number of menstrual cycles \([\text{before WSD: 7.3 ± 0.6 cycles; after WSD: 9.2 ± 0.4 cycles;} t(11) = -2.99, P = 0.01] \). There was also an increased number and percentage of putative ovulatory cycles (as determined by elevated P₄ during the luteal phase) from before to after onset of WSD \([\text{before WSD: 49.6 ± 8.7% ovulatory; after WSD: 86.2 ± 2.8% ovulatory;} t(11) = -4.62, P = 0.001] \). T-treated and control animals did not differ in the number of menstrual cycles (T treated, 14.2 ± 0.9 cycles; control, 13.8 ± 0.6 cycles) or in the number or percentage of ovulatory cycles (T treated, 84.7 ± 4.9% ovulatory; control, 87.7 ± 3.1% ovulatory) that occurred when they were 5.5–7 yr of age and on the WSD.

Samples were taken daily throughout one entire menstrual cycle after the animals had been on the WSD for 14 mo, and each animal experienced an ovulatory cycle as indicated by elevated P₄ levels. There was no overall effect of 14 mo of WSD on peak E₂ during the follicular phase, nor on peak P₄ or P₄ AUC during the luteal phase when all of the animals were considered \((6 \text{ T treated and 6 control}) \). This was still true when only analyzing animals that ovulated \((4 \text{ T treated and 5 controls ovulated before WSD; all animals ovulated 14 mo after WSD}) \). However, in animals that were ovulatory both before and after WSD, there was a significant decrease in D1–3 E₂ values after 14 mo on the WSD \([\text{before WSD: T treated, 78.2 ± 18.0 pg/ml; control, 90.2 ± 12.0 pg/ml; 14 mo WSD: T treated, 53.4 ± 10.7 pg/ml; control, 49.8 ± 6.1 pg/ml; } F(1,7) = 5.57, P = 0.05; \) Fig. 4], and a significant decrease in E₂ surge dynamics (measured by AUC) after 14 mo on the WSD in control females, and T-treated females tended to have reduced E₂ AUC \([\text{before WSD: T treated AUC, 1,879 ± 184.8 pg/ml; control AUC, 2,056 ± 259.4; 14 mo WSD: T treated AUC, 1,205.5 ± 220.6; control AUC 1,289.5 ± 121.3; repeated-measures ANOVA treatment \times \text{year} F(2,10) = 0.025; \) control (before vs. after WSD), \( P = 0.032; \) T treated (before vs. vs. after WSD), \( P = 0.062 \). Cycle length also decreased significantly from before to 14 mo after WSD \([\text{before WSD: T treated, 30.2 ± 2.7 days; controls, 30.6 ± 1.9 days; 14 mo WSD: T treated, 25.8 ± 0.9 days; controls, 26.8 ± 0.6 days; } F(1,7) = 8.66, P = 0.022; \) Fig. 4]. This was due mainly to a decrease in follicular phase length \([\text{before WSD: T treated, 17.0 ± 4.1 days; controls: 15.2 ± 2.5 days; 14 mo WSD: T treated, 11.5 ± 1.2 days; controls: 10.8 ± 0.6 days; } F(1,7) = 4.92, P = 0.062; \) Fig. 4], which mimicked E₂ AUC pattern, since there was no significant change in luteal phase length. Luteal phase length was positively correlated with P₄ AUC \((r = 0.604, P = 0.037) \).

After 14 mo on the WSD, there was a significant treatment effect on peak P₄ value during the luteal phase, with T-treated animals having lower peak P₄ compared with controls \([\text{T treated, 6.8 ± 1.1 ng/ml; control, 12.1 ± 1.6 ng/ml; } t(10) = 2.0, P = 0.02; \) Fig. 4]. T-treated animals also had a lower AUC when P₄ values were assessed during the luteal phase \([\text{T treated, 32.7 ± 5.4 ng/luteal phase; control, 52.4 ± 6.0 ng/}}]

**Fig. 2.** Number (A) and amplitude (B) of luteinizing hormone (LH) pulses occurring in testosterone (T)-treated and control animals during a 12-h period on days (D) 1–3 of a menstrual cycle before, 6 mo after, and 14 mo after starting the WSD. Data from before WSD were previously published \((59) \). Closed bars, T-treated animals; open bars, control animals. *Significantly different from before WSD \((P < 0.05) \).
luteal phase; $r(10) = 2.45, P = 0.034]$. There were no group differences in $E_2$ values measured on D1–3, peak $E_2$ during the follicular phase, FSH measured on D1–2, the LH–FSH ratio on D1–2, follicular phase length, luteal phase length, or total cycle length (all $P > 0.1$). These results did not change when controlling for weight.

When analyzing the ovarian ultrasound data from the early follicular phase from before to 14 mo after beginning the WSD, there was an increase in total numbers of antral follicles ($F(2,20) = 45.8, P < 0.001$; Fig. 5A) and a decrease in maximum follicle size ($F(2,20) = 7.92, P = 0.003$; Fig. 5B) in both groups, but there was no diet $\times$ treatment interaction and no main effect of T treatment. However, after 14 mo on WSD, T-treated females displayed more antral follicles per ovary than the controls during the late follicular phase ($F(1,10) = 6.38, P = 0.027$; Fig. 6A). Additionally, the size of the largest antral follicle was smaller in T-treated females compared with controls during the late follicular phase ($control, 2.1 \pm 0.3 \text{ mm}; T \text{ treated}, 1.4 \pm 0.1 \text{ mm}; F(1,10) = 4.84, P = 0.053$; Fig. 6B). There was also a significant correlation between percent weight gained on WSD and the total number of antral follicles present on ovaries during the late follicular phase ($r = 0.425, P = 0.039$; Fig. 6C). There were no effects of time or T treatment on ovarian circumference ($P > 0.1$).

Experiment 3. Metabolic Parameters

When GTT data were compared between before and 12 mo after onset of WSD, there was a significant increase in peak glucose [$F(1,10) = 8.0, P = 0.018$], baseline (fasting) insulin [$F(1,10) = 27.1, P < 0.001$; Fig. 7D], peak insulin [$F(1,10) = 8.41, P = 0.016$], and AIRg [$F(1,10) = 10.2, P = 0.011$]. However, there was no main effect of WSD on SI ($P = 0.61$) or DI ($P = 0.29$). Weight, percent fat, percent central fat, and changes in these variables while on the WSD were not correlated with any parameters measured using the GTT.

After 12 mo on the WSD, there were no treatment effects on baseline or peak glucose, baseline or peak insulin, or on AIRg or Sg as calculated by MINMOD (all $P > 0.1$, data not shown). However, T-treated animals displayed lower SI [T treated, $5.3 \pm 1.8 \text{ (mU/l)}^{-1} \text{/min}; control, 13.9 \pm 3.4 \text{ (mU/l)}^{-1} \text{/min}; r(10) = 2.23, P = 0.05$; Fig. 7A]. There was also a significant difference in DI, the unitless product of SI and AIRg, with T-treated animals having a lower DI [T treated, $4,223 \pm 791$; control, $7,567 \pm 1,099$; $r(10) = 2.47, P = 0.03$; Fig. 7B]. These differences remained significant when controlling for weight.

Luteal phase length when the animals had been on the WSD for 14 mo correlated positively with SI ($r = 0.909, P < 0.001$). SI was also positively correlated with $P_4$ AUC ($r = 0.69, P = 0.014$). There were no significant correlations between SI and follicular phase length, D1–3 $E_2$, peak $E_2$, or peak $P_4$ (all $P > 0.1$).

There was a trend toward a main effect of time when basal metabolic rate (measured at night between 2300 and 0300) was compared from before with 1 yr after beginning WSD, with metabolic rate decreasing over this time period [$F(1,10) = 4.192, P = 0.068$]. There were no treatment effects on basal metabolic rate either before or after WSD consumption (before WSD: T treated, $1.50 \pm 0.16 \text{ kcal/kg}^{-1} \cdot \text{h}^{-1}$; control, $1.84 \pm 0.37 \text{ kcal/kg}^{-1} \cdot \text{h}^{-1}$; 1 yr WSD: T treated, $1.21 \pm 0.08 \text{ kcal/kg}^{-1} \cdot \text{h}^{-1}$; control, $1.23 \pm 0.05 \text{ kcal/kg}^{-1} \cdot \text{h}^{-1}$, $P = 0.8$). There were also no treatment effects on metabolic rate measured over the rest of the 24-h period (all $P > 0.1$, data not shown).

There was a significant effect of diet on body composition, since percent fat and percent central fat increased significantly
and percent lean mass decreased significantly during 16 mo of WSD consumption (all \( P < 0.001 \); Table 1). There was no effect of T treatment on percent fat, percent central fat, or percent lean mass in monkeys before or 16 mo after starting the WSD.

DISCUSSION

The focus of this study was to examine the effects of chronic HA, with introduction of a WSD, on metabolic and reproductive parameters in female primates. The results may be relevant to considering the effects of higher-than-normal androgens in producing the PCOS phenotype in women, but not the ultimate (e.g., potentially genetic) causes of HA. The findings presented in this paper indicate that mildly elevated levels of androgen, coupled with consumption of a typical WSD, can lead to functional disturbances in the neuroendocrine, ovarian, and metabolic systems. After being on the WSD for 16 mo, all animals showed increases in body weight and percentage body fat, as expected. While on the WSD and over the course of development, the central neuroendocrine drive to the reproductive axis in the control (WSD alone) animals increased so that it was similar to that in the T-treated animals, indicating that T treatment led to earlier maturation of the hypothalamic pulse generator. However, during the period on WSD, LH pulse amplitude was diminished both with and without T treatment. The size and dynamics of the antral follicle pool in the ovaries were also altered by the WSD both with and without T exposure. T-treated monkeys also had significantly lower SI compared with controls, suggesting that T in combination with increased adiposity had a negative effect on metabolic function.

While consuming a WSD, there were no differences in early follicular phase LH pulse frequency or LH pulse amplitude between the T-treated and control groups. Compared with measurements taken before animals started the WSD (59), LH pulse frequency increased in control animals but did not change in T-treated monkeys, such that monkeys from both groups had approximately hourly LH pulses after 6 mo on this diet. Women with PCOS, who have elevated androgens, consistently have about 1 LH pulse/h (86), whereas in healthy women, LH pulse frequencies during the early follicular phase have been reported to be 1 pulse/90 min (4, 86) in some studies and 1 pulse/h in other studies (44, 70). However, most studies that were performed in normally cycling, nonexperimental, female monkeys detected 1 LH pulse/h during the early follic-
obesity was associated with a decrease in LH pulse amplitude and other parameters were only related to weight. Ongoing studies, with control and T-treated monkeys on both normal diet and WSD throughout development, will be able to address these issues.

Neither LH responsiveness to GnRH nor the effect of P₄ negative feedback on pulsatile LH secretion was different between the T-treated and control groups during consumption of the WSD. Decreased sensitivity to P₄ feedback is seen in about one-half of PCOS patients and HA adolescents (22, 68), and has also been reported in adult female sheep that were treated prenatally with T (72). Visual inspection of the current data (see Fig. 3) reveals that there are several animals that did not have a decrease in pulse number between D1–3 and the luteal phase of a menstrual cycle; however, these animals are distributed between the T-treated and control groups, so decreased sensitivity to P₄ negative feedback was not more prevalent in T-treated compared with control animals. It is also unlikely that there was an effect of the increased adiposity from the WSD on the sensitivity of the hypothalamic pulse generator to P₄ negative feedback, since the animals that had no change in LH pulse frequency were not the animals that gained the most weight or had the highest percent fat after switching to the WSD (data not shown). It is possible that the reduced sensitivity to P₄ feedback seen in PCOS patients is not due to the direct actions of T, but is instead caused by entrainment of the hypothalamic-pituitary axis during development, which could explain why we did not see group differences in this study.

Changes in ovarian structure and function were apparent in both the control and T-treated groups after 14 mo of WSD. The animals had shorter follicular phases, which were accompanied by lower levels of E₂ on D1–3 of the menstrual cycle. Low E₂ during the follicular phase is associated with luteal phase defects (LPD) in women (26), and the current data showing that T-treated monkeys on WSD also had lower P₄ levels in the luteal phase suggest that androgen exacerbates these abnormalities. Notably, the reduced E₂ levels in the early follicular phase suggest that the modest levels of exogenous T do not serve as a major substrate for estrogen production. This was tested during our initial studies (Ref. 59, unpublished data) before introducing the WSD protocol. Measurement of circulating E₂ before and after T implantation indicated no differences. This does not rule out some elevation in local E₂ in ovarian compartments where aromatization occurs, especially in the dominant follicle. However, no differences in E₂ levels were noted between treatment groups at midcycle. Future studies will also examine the effects of both WSD and T on steroidogenesis within the ovary/dominant follicle.

Additionally, the monkeys displayed an increased number of small antral follicles and a decrease in antral follicle size during the early follicular phase after WSD consumption. Alterations in follicular dynamics were also evident during the late follicular phase, when changes were more pronounced in the T-treated group. We previously used ultrasound studies to document changes to the antral follicle cohort during the follicular phase in rhesus macaques (12). In adult rhesus females on a normal diet with normal menstrual cycles, the largest antral follicle is typically larger (4.36 ± 0.63 mm)
during the late follicular phase than was found in either the control or T-treated group during the current study (see Fig. 6B). Additionally, our previous study found that the dominant follicle had a cohort of only three to four other antral follicles during the late follicular phase (12) compared with the average cohort of seven follicles found in this study (Fig. 6A). Androgen exposure was previously shown to increase antral follicle formation when given in high doses to adult rhesus monkeys for 3–10 days (85). Our study indicates that long-term, mildly increased androgen exposure compounded the effect of the WSD to alter follicular growth dynamics, either delaying or suppressing the growth of the dominant antral follicle and increasing the number of small antral follicles present.

After 14 mo on the WSD, T-treated animals had a lower peak P₄ value and lower P₄ AUC during the luteal phase compared with control animals, which could have resulted from defects in the dominant antral follicle, as discussed above. Low levels of P₄ during the luteal phase in women are indicative of LPD (25, 77). LPD have been associated with abnormal gonadotropin secretion (25, 40, 77, 78), poor follicular quality (26), and stress during the follicular phase of the menstrual cycle (89). Women with PCOS were found to have lower levels of P₄ than controls during the luteal phase after either spontaneous or induced ovulation, indicative of the presence of LPD in these women (60). Similarly, Goy and Robinson (36) reported an increase in LPD in female monkeys that were treated prenatally with T also had increased abdominal fat mass compared with weight- and BMI-matched controls (30). Thus, androgens are likely the cause of increased central fat, as opposed to the increased central adiposity leading to increased levels of androgens. We therefore expected the T-treated monkeys in the current study to have higher levels of central adiposity compared with the control animals, but this was not the case. It is possible, however, that, if the animals remained on the WSD for a longer period of time and continued to gain weight, a group difference might have emerged.

Total postpubertal weight gain was higher in T-treated animals compared with controls. Although T-treated monkeys did not eat more calories than control animals, there was a strong trend toward the T-treated animals being less active than controls during the last 10 mo of this study. Studies in humans
and animals found that individuals that are less active tend to gain more weight (17, 47, 82), which is consistent with the current data. Because activity levels were not assessed in our prior study (59) before T implants were placed, it is unknown if the T-treated animals had lower levels of activity at baseline or whether the decreased activity levels occurred later as a result of chronic T treatment.

Metabolic changes were also evident between the control and T-treated groups after 12 mo on the WSD. SI scores were significantly lower in T-treated compared with control animals, even after controlling for weight. Similarly, women with PCOS have lower SI than weight-matched controls, and this relationship is present even in lean PCOS patients (20, 28). However, we did not find any effect of T on SI when the monkeys were tested at 3.5 yr of age, before starting the WSD. This suggests that, while initial T treatment alone did not lead to decreased SI in this study, T treatment in combination with adiposity had a more detrimental effect on SI than adiposity alone. Alternatively, more prolonged T treatment until 6.5 yr of age may have led to decreased SI in the absence of WSD consumption. The trend toward increased insulin levels during WSD exposure is consistent with those observed in rhesus females with ~20% body fat in the ONPRC colony (54). Unfortunately, there is a paucity of data from age-matched “lean” (3% fat) females as young adults. Ongoing studies using larger cohorts of female monkeys with and without WSD should clarify these data.

Studies by Abbott and colleagues (1) observed insulin resistance and hyperinsulinemia in all monkeys they studied that had a high BMI (over 41.2 kg/m²) in adulthood. However, animals that were prenatally androgenized in addition to being obese and insulin resistant were more likely to be anovulatory than animals that were obese but not exposed to T during gestation. The average BMI after 16 mo on the WSD in the current study was 25.6 kg/m², and the highest was 34.6 kg/m², so it is possible that some of the T-treated animals may have become anovulatory at a later time if they continued to gain weight on the WSD.

Although we did not see any effect of T treatment on ovulation rates in the current study, there was a relationship between metabolic and ovarian parameters. The animals with lower SI also had shorter luteal phases, which can be an early indicator of LPD (77). Decreased SI and anovulation are both common in women with PCOS, and, when these women ovulate, they often have LPD (60). Thus, it is interesting that the animals with decreased SI were the same animals that had shorter luteal phases in this study, and it is possible that the T-treated monkeys would develop higher rates of anovulation with continued exposure to elevated T.

Table 1. Body composition parameters before and 16 mo after starting WSD

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<td></td>
<td>Weight, kg</td>
<td>Fat, %</td>
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<td>Lean mass, %</td>
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<td>Control</td>
<td>4.6 (0.2)</td>
<td>1.5 (0.2)</td>
<td>0.6 (0.1)</td>
<td>95.4 (0.2)</td>
<td>5.9 (0.3)</td>
<td>14.9 (2.8)</td>
<td>16.3 (3.9)</td>
<td>81.3 (3.3)</td>
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<tr>
<td>T treated</td>
<td>5.2 (0.1)</td>
<td>1.7 (0.3)</td>
<td>1.0 (0.3)</td>
<td>95.2 (0.3)</td>
<td>6.7 (0.4)</td>
<td>19.3 (3.8)</td>
<td>22.2 (4.9)</td>
<td>76.0 (3.6)</td>
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Data are presented as mean (SE). T, testosterone. There was a significant change in all parameters from before to after Western style diet (WSD), although there were no between-group differences.
There was a decrease in basal metabolic rate from before to 12 mo after the animals began eating the WSD. This was likely due to aging, since metabolic rate declines during puberty in people (16) and continues to decline with age (37, 71, 73). The decrease in metabolic rate also could have resulted from a decrease in activity, since lower activity is associated with a decreased metabolic rate (52, 69). The lack of differences in metabolic rate between T-treated and control animals indicates that the change in metabolic rate over time was likely not attributable to T.

Obesity was associated with increased levels of free T in women in some (31, 79, 83), but not all (14), studies. We did not see an increase in total T or free T, or a decrease in SHBG in the monkeys as they gained weight on the WSD. Thus, the effects of WSD that we report in control monkeys (i.e., weight gain and changes in ovarian structure) are unlikely to be a result of increased circulating androgen.

In summary, although increased adiposity as a result of the WSD did not appear to act in concert with T treatment to aggravate neuroendocrine dysfunction, T + WSD treatment reduced SI, increased the number and reduced the maximal size of antral follicles at midcycle, and reduced P4 secretion during the luteal phase of the menstrual cycle compared with controls. Thus, prolonged elevation of T combined with WSD may lead to impairment of metabolic and ovarian function. This dysfunction may have resulted from the extended length of T treatment, or the combination of T and WSD. However, since this project was a preliminary study, resources were not available to implement the optimal experimental design, which would have included both control and T-treated groups that were fed normal monkey chow, as well as control and T-treated groups that received WSD. Without these non-WSD groups, it is difficult to definitively state the role of the WSD (vs. age and length of T treatment) with regard to changes in SI and ovarian function in the T-treated group in the current study. It may have been that T treatment alone for a prolonged period would have led both to luteal dysfunction and reductions in SI. Ongoing studies that include groups of both T-treated and control monkeys maintained on both normal monkey chow and WSD should clarify the role of adiposity compared with the role of elevated androgen on the neuroendocrine, reproductive, and metabolic parameters that were measured in the current study, which may have relevance to the PCOS phenotype in women.

ACKNOWLEDGMENTS

We thank Mandi Bulechowsky for outstanding technical assistance and express appreciation to the ONPRC Department of Comparative Medicine, veterinary and surgical staff for the excellent care of the animals in this study. Gratitude is expressed to the ONPRC Endocrine Technology Support Core Laboratory and the Assay Core of the Center for Reproductive Physiology at the University of Pittsburgh for assistance with hormone assays. We also thank Dr. Elinor Sullivan for consultations regarding metabolic measurements made in this study.

GRANTS

This study was funded by a grant from the OHSU ARCS Foundation and NIH Grant T32-HD-07133. A fellowship from the OHSU ARCS Foundation and NIH Grant T32-HD-07133.

DISCLOSURES

The authors have nothing to disclose.

AUTHOR CONTRIBUTIONS


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