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High-Fat, High-Sugar Diet Disrupts the Preovulatory Hormone Surge and Induces Cystic Ovaries in Cycling Female Rats

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Diet-induced obesity has been associated with various metabolic and reproductive disorders, including polycystic ovary syndrome. However, the mechanisms by which obesity influences the reproductive system are still not fully known. Studies have suggested that impairments in hormone signaling are associated with the development of symptoms such as acyclicity and ovarian cysts. However, these studies have often failed to address how these hormonal changes arise and how they might contribute to the progression of reproductive diseases. In the present study, we used a high-fat, high-sugar (HFHS) diet to induce obesity in a female rodent model to determine the changes in critical reproductive hormones that might contribute to the development of irregular estrous cycling and reproductive cycle termination. The HFHS animals exhibited impaired estradiol, progesterone (P4), and luteinizing hormone (LH) surges before ovulation. The HFHS diet also resulted in altered basal levels of testosterone (T) and LH. Furthermore, alterations in the basal P4/T ratio correlated strongly with ovarian cyst formation in HFHS rats. Thus, this model provides a method to assess the underlying etiology of obesity-related reproductive dysfunction and to examine an acyclic reproductive phenotype as it develops.

Freeform/Key Words: decreased luteinizing hormone, hormone surge, increased estradiol, obesity, ovarian cysts, testosterone

Obesity has been associated with reproductive dysfunction and infertility in females [1, 2]. For example, obese women show a high rate of anovulation, infertility, and miscarriage [3–9]. Furthermore, obesity has been implicated in the development of reproductive disorders such as polycystic ovary syndrome (PCOS), which is the most common cause of infertility among women [10–12]. As many as 38% to 88% of PCOS patients have been reported to be obese [13]. PCOS and obesity often share manycomorbidities, such as hyperinsulinemia, leptin and insulin resistance, and other metabolic disruptions [14–17]. Moreover, obesity further exacerbates these symptoms in women with PCOS [18–20].

However, although a clear connection exists between obesity and reproductive impairment, the underlying mechanisms are unclear. In our previous study, we used a high-fat, high-sugar (HFHS) diet to induce obesity in rats and demonstrated the appearance of multicystic ovaries and disrupted estrous cyclicity, both indicators of reproductive impairment [21]. The diet, administered after weaning, provides a method to examine the natural progression of these adverse reproductive impairments as they develop. Therefore, our HFHS

Abbreviations: AMH, anti-Müllerian hormone; CL, corpora lutea; E2, estradiol; FSH, follicle-stimulating hormone; GF, Graafian follicle; HFHS, high-fat, high-sugar; LH, luteinizing hormone; P4, progesterone; PCOS, polycystic ovary syndrome; PVC, persistent vaginal cornification; T, testosterone.

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model of diet-induced obesity provides a unique opportunity to determine the underlying etiology of obesity-associated reproductive dysfunction.

Studies have shown that aberrant hormone signaling is associated with reproductive disorders and cycle irregularity [22–24]. One critical reproductive hormone, luteinizing hormone (LH) is a gonadotropin released from the pituitary shortly before ovulation. The preovulatory release of LH during the reproductive cycle serves as the principal signal to initiate ovulation. Reproductive dysfunction and anovulation have thus often been associated with the absence of LH surges and/or abnormal basal levels of LH [25–28]. In addition, one study of mice showed hypothalamic dysfunction (hypogonadotropic hypogonadism) specifically as a result of diet-induced obesity [29], and another study characterized attenuated LH release in leptin-resistant obese rats [30].

Furthermore, the ovarian steroid hormones progesterone (P4) and estradiol (E2) exert feedback effects on LH release. In normal females, the midcycle LH surge occurs as a result of a switch from E2-negative to E2-positive feedback [31–33]. This feedback has been shown to be dependent on P4, and impairments in P4 and E2 signaling have been associated with infertility and anovulation [24, 25, 34–37]. Several studies have found impaired sex steroid hormone feedback and altered LH release in models of reproductive disorders [38–40]. Additional studies have shown altered sex steroid levels in obese females [41, 42]. Adverse effects of obesity on ovarian morphology have also been demonstrated; however, it is still not fully understood how these effects might arise, in particular in conjunction with altered hormone levels [9, 43].

Therefore, in the present study, we used a HFHS diet to investigate the mechanisms by which diet-induced obesity initiates physiological changes that could lead to parallel changes in both ovarian morphology and hormone levels. We hypothesized that animals consuming the HFHS diet would experience impaired reproductive function through alterations in hormone levels during the preovulatory surge and subsequent abnormal ovarian morphology. To the best of our knowledge, this is the first study to report hormone levels during the proestrus surge that correspond with cystic ovarian morphology in a diet-induced obesity model.

1. Materials and Methods

A. Animals

Female Sprague Dawley rats (n = 30; P17) were obtained from Charles River (Raleigh, NC) and were housed with their mothers until weaning at day 23. At weaning, the rats were randomly assigned to either the HFHS diet (n = 16) or a control diet (n = 14) group. The control group had *ad libitum* access to water and standard rat chow (3.1 kcal/g, 17% calories from fat; LM-485; Envigo, Indianapolis, IN). The HFHS diet group had *ad libitum* access to water, a 32% sucrose solution, and high-fat chow (5.24 kcal/g, 60% of calories derived from fat; D12492; Research Diets, New Brunswick, NJ). The rats were housed in pairs in a climate-controlled environment with a 12-hour/12-hour light/dark cycle (lights on at 8 AM). The rats were weighed every other day throughout the study. The Washington and Lee University institutional animal care and use committee approved all animal procedures (protocol no. NT0717).

B. Estrous Cycle Monitoring

Estrous cycles were monitored by daily vaginal lavage during weeks 8 to 14 of the diet. Estrous cycle stages were identified via cytological examination, as previously described [44]. In brief, if leukocytes were a dominant cell type, the sample was identified as diestrus. If nucleated cells were abundant, the sample was considered proestrus. Samples with mostly cornified cells were classified as estrus. Large non-nucleated cells were an indicator of persistent estrus. The duration of each estrous cycle was determined as the number of days before the appearance of each diestrus phase. The rats were identified as demonstrating

persistent vaginal cornification (PVC) if the smears revealed ≥5 consecutive days spent in the estrus phase of the cycle. The identification of PVC has been characterized in previous rodent models of PCOS and in aging animals with reproductive impairment [45, 46]. To calculate the total time spent in each phase of the cycle, we divided the total number of days spent in each specific phase (proestrus, estrus, and diestrus) by the total duration of the sampling time. Comparisons of the difference in cycle distribution of each stage of the cycle between the two diet groups were evaluated using a χ^2 test. Differences were considered statistically significant at $P < 0.05$.

C. Jugular Vein Cannulation and Blood Samples

After 14 weeks of the diet, the rats underwent jugular cannulation on the morning of proestrus. Under isoflurane anesthesia, a catheter (MRE-040, 0.040-in. outer diameter × 0.025-in. inner diameter, Micro-Renathane; Braintree Scientific, Inc., Braintree, MA) was inserted through the external jugular vein into the right atrium, secured subcutaneously, and exteriorized at the back of the rat, as previously described [47, 48]. On the morning of proestrus, an extension of the catheter tubing filled with saline was connected to the jugular catheter, and the rats were left undisturbed in their cages. Blood samples of 300 μL were withdrawn into plastic heparinized syringes, and the same volume of sterile saline (0.9% NaCl; Teknova, Hollister, CA) was injected through the catheter immediately after removal of each blood sample.

D. Glucose Tolerance Testing

Glucose tolerance testing was performed to assess for hyperinsulinemia and insulin resistance. Before testing, the rats were fasted overnight to deplete glycogen stores and reduce baseline variability between subjects. Blood samples were obtained either via tail nick or from the indwelling jugular catheter. Basal blood glucose levels (mg/dL) were measured using an Accu-Chek Compact Plus whole-blood glucose monitor. The rats immediately received a bolus dose of glucose (1 g/kg) via intraperitoneal injection, and additional blood samples were collected at 5, 15, 30, 60, and 90 minutes after injection. To examine the insulin levels, blood was collected at the same time points, allowed to clot, and centrifuged to obtain serum. Serum samples were then stored at −20°C until the insulin levels were determined using a rat insulin enzyme-linked immunoassay kit as per the manufacturer's instructions (model 90010; Crystal Chem).

E. Reproductive Hormone Measurements

Reproductive hormone assays were performed by the University of Virginia Ligand Core Facility. Serum LH and follicle-stimulating hormone (FSH) were measured using a rat multiplex assay (reportable range, 0.24 to 30.0 ng/mL and 2.4 to 300.0 ng/mL, respectively). Serum T, E2, and P4 levels were measured using a radioimmunoassay (range, 10.0 to 1600.0 ng/mL, 3.0 to 300.0 pg/mL, and 0.15 to 80.0 ng/mL, respectively). Anti-Müllerian hormone (AMH) was measured using an enzyme-linked immunoassay assay according to the manufacturer's instructions (model CSB-E11162r; CUSABIO).

F. Perfusion and Tissue Collection

On the morning of diestrus I, the rats were deeply anesthetized with isoflurane, blood was collected through cardiac puncture, and the rats were transcardially perfused with saline and 4% paraformaldehyde. Visceral fat pads were collected and weighed immediately after perfusion. Upper trunk fat in females has been associated with metabolic and reproductive disruption [49–52]. In particular, visceral fat deposits have been associated with detrimental metabolic and reproductive states [53, 54]. Furthermore, Malafaia *et al.* [55] showed that the deposition of fat in rats changes with the onset of obesity, such that obesity is associated with an increase in upper retroperitoneal fat specifically. Therefore, we separated visceral fat pads

by retroperitoneal fat and parametrial fat to represent the upper abdominal and gonadal fat, respectively. Brains and ovaries were collected and fixed in 4% paraformaldehyde at 4°C overnight before being transferred to a 30% sucrose solution in phosphate-buffered saline. The ovaries were then cleaned by removing the oviduct and any surrounding adipose tissue, weighed, and embedded in optimal cutting temperature matrix (23-730-571; Fisher) for storage at −80°C until cryostat sectioning.

G. Ovarian Histologic and Follicular Assessment

Follicular development was assessed using one ovary from each rat. The ovaries were serially sectioned into 10-μm-thick sections, with every fifth section collected on gelatinized glass slides. Histological examination of tissue sections was conducted after hematoxylin and eosin staining. The total numbers of cysts and corpora lutea (CL) follicle types for each rat were identified according to the criteria, as previously described [45, 56, 57]. In brief, cysts were identified as those follicles displaying a large antral space surrounded by an enlarged and densely stained thecal cell layer and lacking an ovum. CL were identified according to tissue density and the presence of a grainy luteinized cell appearance. CL were counted only when they exhibited more than a 60% area with a higher tissue density. Graafian follicles (GFs) were identified by the presence of the following criteria: distinct antral cavity; a thick, homogenous layer of granulosa cells; and a thin, defined outline of thecal cells. Additionally, the ovum had to be tightly bound to the granulosa layer, and the follicle had to have an ovular shape. The number of follicular structures per ovarian tissue section was then quantified by dividing the total number of follicular structures by the number of sections for that ovary.

H. Statistical Analysis of Histological and Physiological Measurements

Long-term noncycling rats (n = 4) were removed from the reproductive hormone data analysis on proestrus and formed a separate group for hormone analysis. Our exclusion criteria included ≥3 weeks of persistent estrus (PVC) before jugular cannulation. Using these criteria, we excluded three rats from the HFHS group and one from the control group, because these animals were not in proestrus when cannulated. For comparisons between groups, all results are expressed as the mean ± standard error of the mean. The comparison of the mean values between two groups was evaluated using a Student's one-sided *t* test. Differences were considered statistically significant at $P < 0.05$. The statistical significance of all correlations was assessed using linear regression analysis.

2. Results

A. HFHS Diet Increases Weight Gain and Abdominal Fat

To determine the effects of the HFHS diet on weight gain, the rats in both groups were weighed every other day after starting the diet at weaning. At the end of 14 weeks of the diet, the HFHS rats weighed substantially more than did the controls (368.88 ± 11.07 g vs 304.79 ± 6.42 g; $P = 4.32E-07$; Fig. 1). A substantial difference in weight gain between the two groups was achieved by the third week of diet exposure, and the HFHS rats continued to gain significantly more weight than did the controls for the remainder of the study period [Fig. 1(a)]. Furthermore, because abdominal fat has been associated with reproductive and metabolic disorders [10–12], we measured the amount of abdominal fat in each rat. The HFHS rats exhibited a significantly greater percentage of abdominal fat relative to body weight (5.36% ± 0.39%) compared with controls [2.38% ± 0.21%; $P = 5.82E-07$; Fig. 1(b)]. Thus, the HFHS diet alone was successful in inducing obesity.

B. HFHS Diet Increases Estrous Cycle Irregularities

As obesity has been associated with anovulation and irregular cycling [2, 58, 59], we monitored the estrous cycle of our rats every day after the first vaginal opening, from weeks 8 to 14

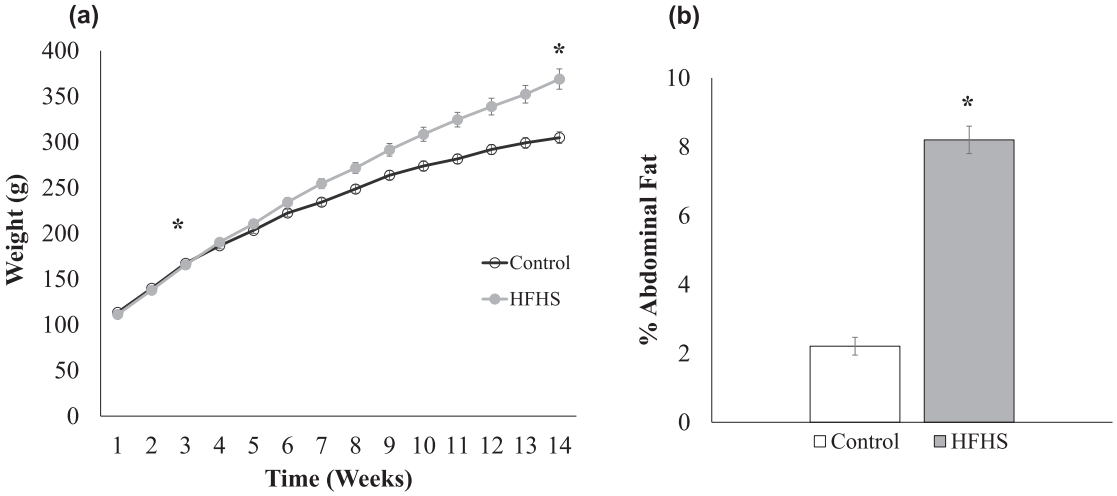

Figure 1. HFHS diet increases body weight and abdominal fat. (a) Mean HFHS and control animal body weights by weeks of diet exposure. (b) Average percentage of abdominal fat weight relative to total body weight. Error bars indicate standard error of the mean; gray circles, HFHS diet group; and open circles, controls.

of the diet. We found that the HFHS group, on average, spent a greater fraction of time in estrus [0.44 ± 0.06 vs 0.28 ± 0.03; Fig. 2(a)], less time in proestrus [0.18 ± 0.02 vs 0.23 ± 0.01; Fig. 2(b)], and less time in diestrus [0.38 ± 0.04 vs 0.49 ± 0.02; Fig. 2(c)] compared with the controls ($P < 0.001$). Furthermore, at the conclusion of our study, one half of the HFHS rats exhibited extended cycles, with multiple periods of persistent estrus.

C. HFHS Diet Increases Fasting Insulin, but not Glucose, Levels

Although no substantial difference was found in the fasting blood glucose levels between diet groups [Fig. 3(d)], the fasting insulin levels in HFHS rats were significantly higher (29.06 ± 3.33 mU/L) than their control diet counterparts [11.81 ± 2.06 mU/L; $P < 0.0002$; Fig. 3(c)]. Additionally, glucose tolerance testing revealed a statistically significant difference in insulin

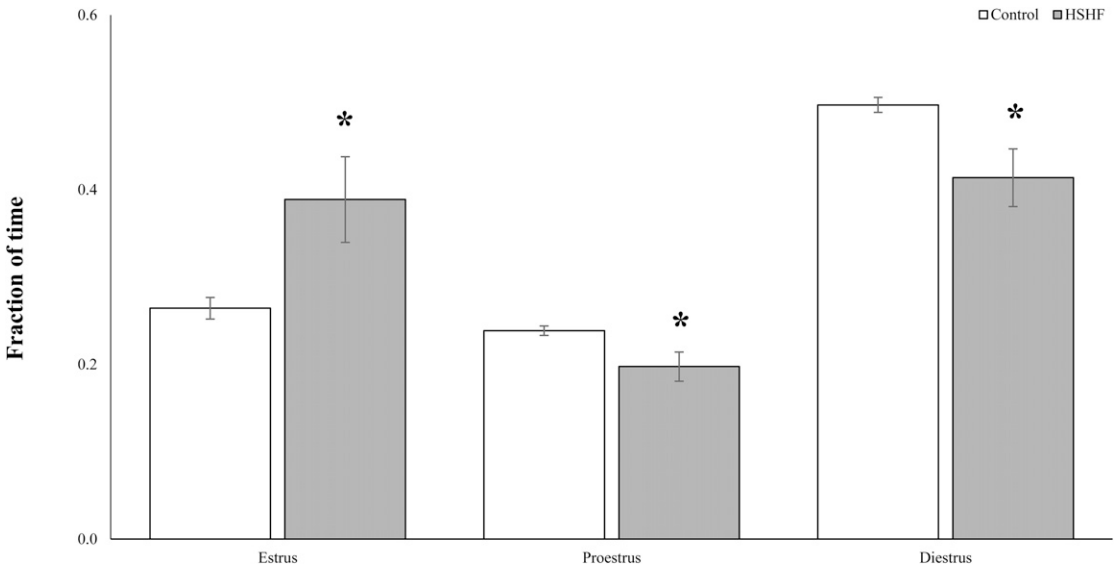

Figure 2. HFHS diet increases irregular cycling. Graph showing fraction of time spent in each stage of the estrous cycle.

area under the curve between control (2052.1 ± 368.1) and HFHS [4069.2 ± 331.4; $P < 0.002$; Fig. 3(b)] rats.

D. HFHS Diet Increases Ovarian Cysts and Decreases CL

In our previous study, we found an increase in follicular cysts and decrease in CL as a result of a HFHS diet [21]. However, in the present study, the rats were maintained on the diet for a longer period (4 additional weeks). To confirm our previous findings, we conducted a

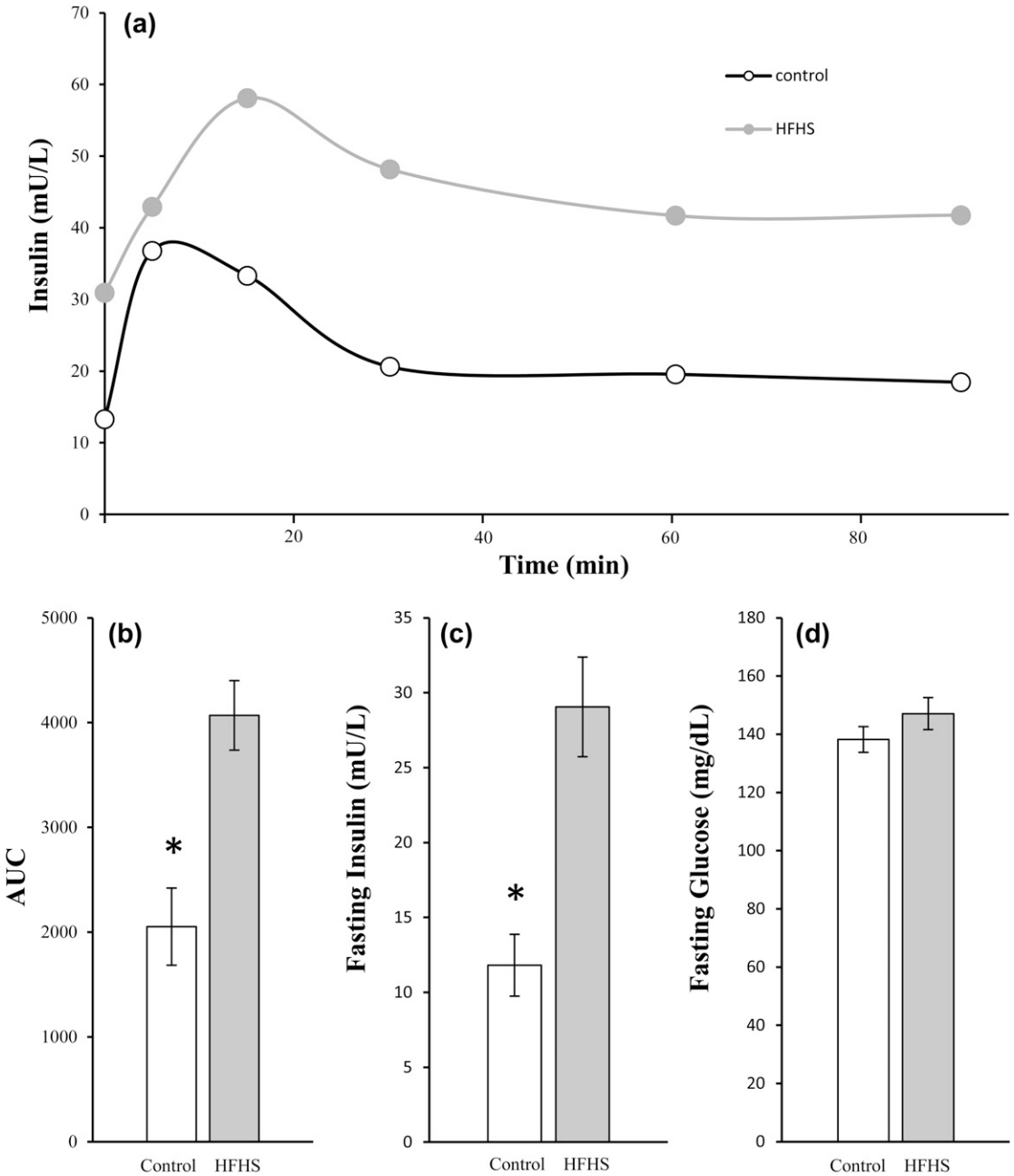

Figure 3. HFHS diet increases fasting insulin, but not glucose, levels. (a) Mean insulin levels over time after a glucose tolerance test in HFHS and control rats. (b) Mean insulin area under the curve (AUC) between control and HFHS rats after a glucose tolerance test. (c) Mean fasting insulin levels between control and HFHS groups. (d) Mean fasting glucose levels in HFHS and control rats.

histological analysis of ovarian tissue to assess any changes in ovarian morphology as a result of the different diets. We found that the HFHS rats had a significantly greater number of cysts (1.89 ± 0.26 cysts per section) compared with the control rats [1.06 ± 0.27 cysts per section; $P = 0.0225$; Fig. 4(c)]. The HFHS rats also exhibited significantly lower CL counts per ovary section (8.95 ± 0.77 CL per section) compared with the controls [12.09 ± 0.92 CL per section; $P = 0.0105$; Fig. 4(d)]. We found no statistically significant difference in GF counts between groups [0.71 ± 0.08 vs 0.54 ± 0.07; $P = 0.075$; Fig. 4(e)], consistent with data from previous studies [60].

E. HFHS Diet Decreases LH on Morning of Diestrus

Because the gonadotropins LH and FSH are critical to follicular development, we examined the levels of these gonadotropins to assess any corresponding changes that might have contributed to the altered ovarian morphology seen on diestrus. Terminal diestrus blood samples were collected and analyzed for serum LH and FSH levels. Although we found no difference in FSH levels between groups [Fig. 5(b)], we found that the HFHS group (1.5 ± 0.3 ng/mL) had a significantly lower LH level on diestrus I compared with the controls [2.4 ± 0.3 ng/mL; $P = 0.025$; Fig. 5(a)], which is consistent with data from the results from studies of obese women [41, 61, 62].

F. HFHS Diet Influences Steroidogenesis Pathway

We also took terminal blood samples to measure serum P4, T, and E2 to assess the changes in steroid hormone levels involved in the steroidogenesis pathway. Although no difference was found in basal P4 or E2 levels, the HFHS group (31.4 ± 1.7 ng/mL) exhibited significantly elevated basal T levels relative to the controls on diestrus I [27.2 ± 1.7 ng/mL; $P = 0.048$; Fig. 6(b)]. Likewise, the noncycling group exhibited higher testosterone levels relative to the controls (29.6 ± 1.4 ng/mL vs 27.2 ± 1.7 ng/mL; $P = 0.045$). Because it is unknown whether this

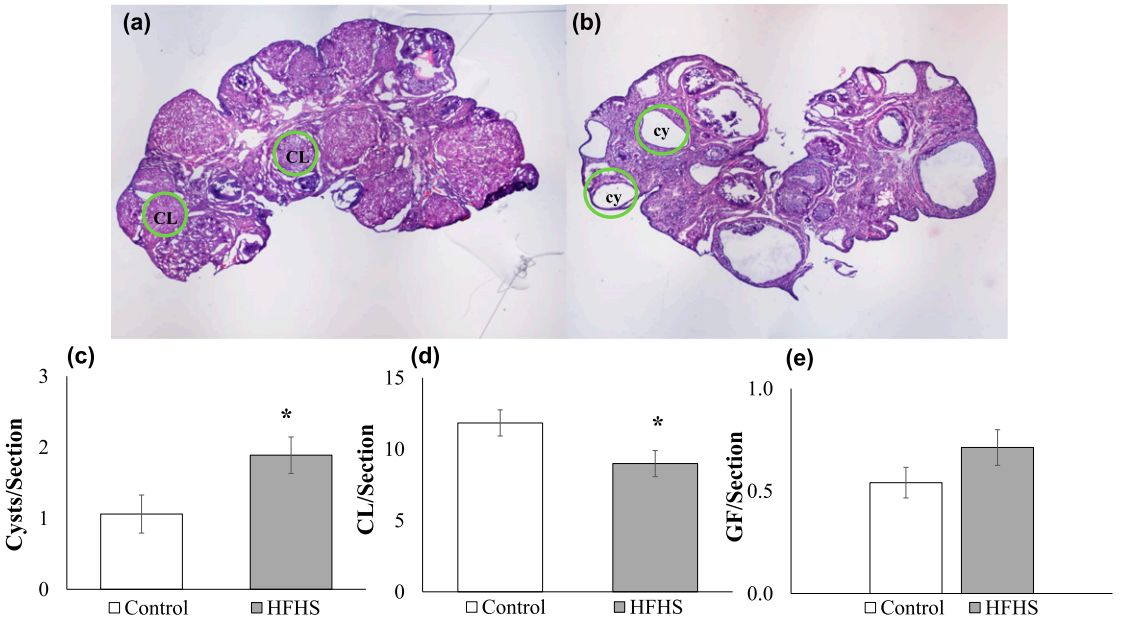

Figure 4. HFHS diet is associated with altered ovarian morphology. (a) Representative photomicrograph of a control ovary. CL are evident throughout the section. (b) Representative photomicrograph of an ovary from a HFHS rat showing numerous fluid-filled cysts (cy) and fewer CL compared with the control ovary. (c) Mean number of cysts per ovary section. (d) Mean number of CL per ovary section. (e) Mean number of GFs per section. Error bars indicate standard error of the mean.

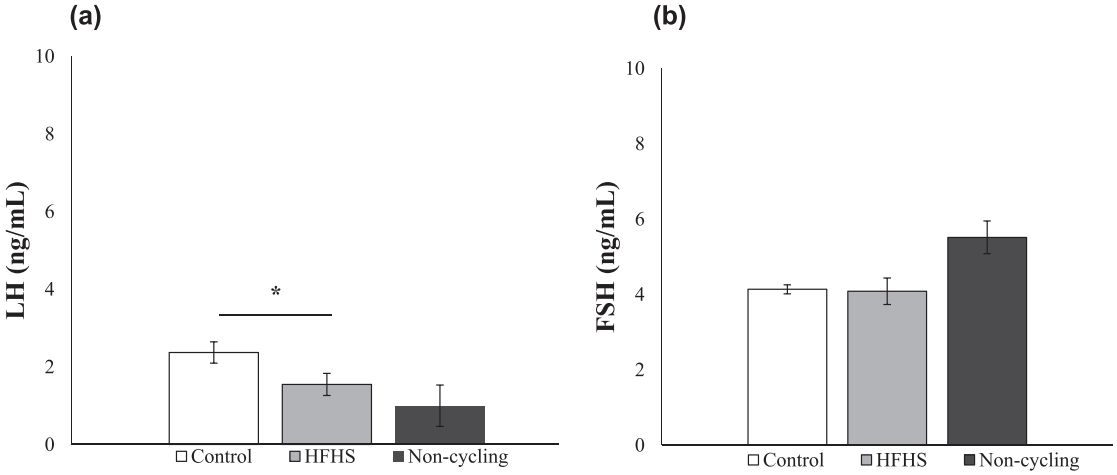

Figure 5. HFHS diet alters LH, but not FSH, levels on diestrus. (a) Mean concentration of LH on diestrus. (b) Mean FSH levels on diestrus. Error bars indicate standard error of the mean.

elevated level of T results from changes in androgen precursor production or reduced conversion of T in a subsequent step, we determined the relative ratios of steroid hormones as sequential conversions in the pathway. No substantial difference was found between the control and HFHS groups for the P4/T and P4/E2 ratios; however, the noncycling group

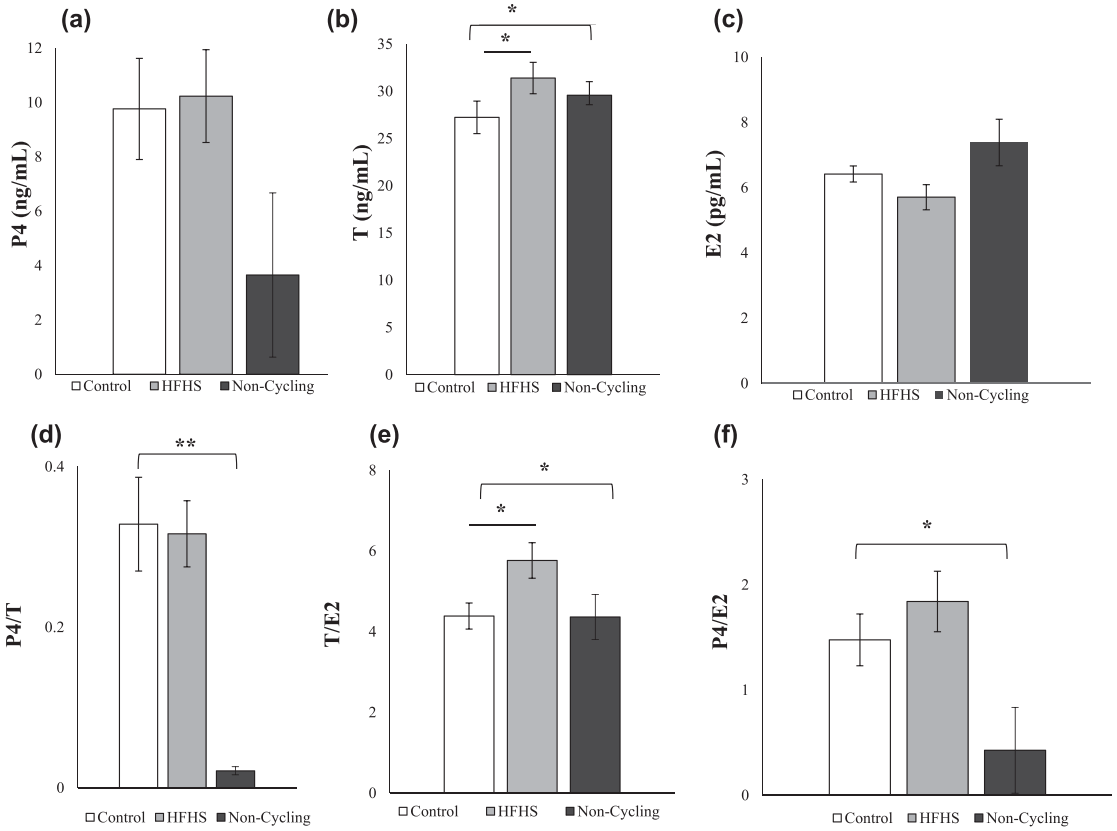

Figure 6. HFHS diet is associated with altered steroidogenesis. (a) Mean progesterone levels of HFHS and control rats on diestrus. (b) Mean T levels of HFHS and control rats on diestrus. (c) Mean E2 levels of HFHS and control rats on diestrus. (d) Mean ratio of P4/T of HFHS and control rats on diestrus. (e) Mean ratio of T/E2 of HFHS and control groups on diestrus. (f) Mean ratio of P4/E2 of HFHS and control groups on diestrus. Error bars indicate standard error of the mean.

had a significantly lower P4/T ratio compared with controls (0.021 ± 0.0049 vs 0.328 ± 0.058; $P = 0.0002$) and a significantly lower P4/E2 ratio than controls [0.427 ± 0.408 vs 1.46 ± 0.246; $P = 0.025$; Fig. 6(d) and 6(f)]. However, when we considered the T/E2 ratio, the final step in the steroidogenesis pathway, we found that the HFHS group had a significantly greater T/E2 ratio (5.76 ± 0.44) than the control group [4.39 ± 0.32; $P = 0.010$; Fig. 6(e)].

G. P4/T Ratio Predicts Formation of Cysts in HFHS Rats on an Individual Level

Because ovaries are the primary site of steroidogenesis, we then determined the relationship between steroid hormone ratios and ovarian structures in our rats. We found that a lower P4/T ratio on diestrus I correlated exponentially with cyst formation in the individual HFHS rats [Fig. 7(a); linear regression, $r^2 = 0.81$; $P < 0.01$). However, no difference was found in the P4/T ratio between the two diet groups [Fig. 6(d)]. Furthermore, we found that a greater P4/T ratio was associated with an increase in CL counts in the HFHS rats [Fig. 7(b); linear regression, $r^2 = 0.45$; $P < 0.01$). These associations were not as strong among the control rats ($r^2 = 0.27$, $P = 0.325$; and $r^2 = 0.085$, $P = 0.541$, respectively; Fig. 7).

H. HFHS Diet Disrupts Hormonal Balance During Preovulatory Surge

Because obesity has previously been linked with anovulation [60, 63], we hypothesized that changes in reproductive hormones during the preovulatory surge would precede the anovulatory state. Therefore, we collected serum blood samples during the preovulatory gonadotropin surge on the evening of proestrus to assess any changes in hormones that contribute to ovulation. We found that the HFHS group exhibited a significantly greater E2 concentration (15.205 ± 3.956 pg/mL) compared with the controls on proestrus at 9 PM [6.655 ± 0.552 pg/mL; $P = 0.027$; Fig. 8(a)]. We found that the LH levels did not reflect the elevated E2 surge, as might be expected during positive feedback. Instead, the HFHS group actually showed a significantly lower LH surge at 9 PM on proestrus (11.7 ± 2.6 ng/mL) compared with the controls [19.0 ± 3.0 ng/mL; $P = 0.042$; Fig. 8(c)]. Furthermore, we found that the HFHS group had a significantly decreased P4 surge at 7 PM on proestrus (23.37 ± 2.94 ng/mL) compared with the controls [35.79 ± 2.34 ng/mL; $P = 0.002$; Fig. 8(b)].

3. Discussion

Diet-induced obesity has been strongly linked with the development of reproductive disorders in females. Previous studies have shown that, along with a variety of metabolic disruptions, diet-induced obesity is associated with impairments in ovarian insulin signaling [64],

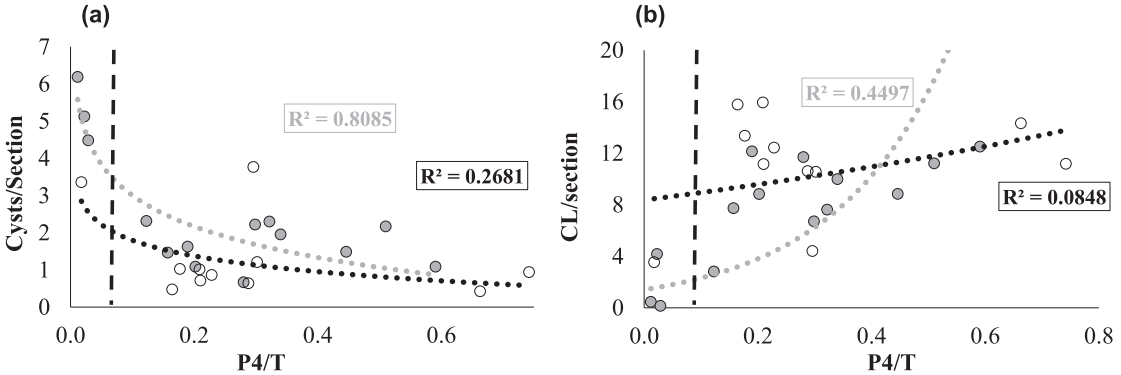

Figure 7. Correlations between cyst and CL counts vs P4/T ratio on diestrus in HFHS and control rats. (a) Plot of individual numbers of cysts vs P4/T ratio on diestrus. (b) Plot of individual numbers of CL vs P4/T ratio on diestrus. Open circles represent individual control rats; exponential black dotted line fitted to controls. Gray circles represent HFHS rats; exponential gray dotted line fitted to HFHS. Vertical black dotted lines denote the separation between cycling (n = 21) and noncycling (n = 4) rats.

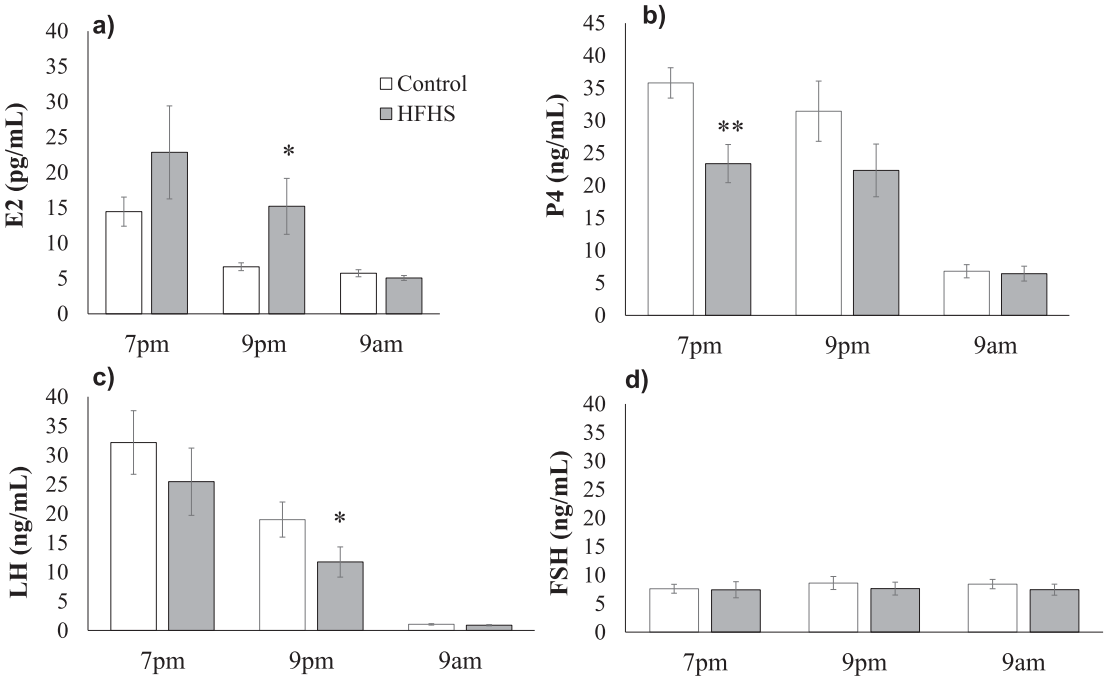

Figure 8. HFHS diet disrupts hormonal levels on proestrus. (a) Mean estradiol levels throughout the preovulatory surge on proestrus. (b) Mean progesterone levels throughout the preovulatory surge on proestrus. (c) Mean LH levels throughout the preovulatory surge on proestrus. (d) Mean FSH levels throughout the preovulatory surge on proestrus. (n = 12 control; n = 13 HFHS).

hormonal imbalances [60], and cycle irregularities [2, 63]. Furthermore, PCOS, the most common cause of anovulatory infertility, is highly prevalent among obese individuals. As many as 38% to 88% of women with PCOS are obese and have hyperinsulinemia, which is often a hallmark of obesity [10, 11, 13, 65, 66]. However, the mechanisms by which obesity itself might contribute to the pathogenesis of acyclicity and/or anovulation are not clearly understood. The present study therefore sought to use an animal model of diet-induced obesity to examine changes in reproductive function as a result of diet.

In the present study, rats were maintained on a HFHS diet for 14 weeks. At the end of the study, HFHS rats exhibited irregular estrous cyclicity, with substantially less time in proestrus and more time in estrus compared with the controls. A greater proportion of time spent in estrus is associated with anovulation, reproductive senescence, and impaired fertility [45, 67–69]. It is possible that the development of prolonged periods of estrus is a consequence of disrupted hypothalamic activity, as suggested in several studies [69, 70]. In work done with aged female rats, a state of constant estrus was also associated with an elevated level of circulating E2 and the development of polycystic ovaries, mirroring the characteristics of the HFHS rats in our study [71, 72]. Additionally, our HFHS rats exhibited significantly elevated insulin levels, which are often characteristic of obesity and have been associated with reproductive malfunction [73–75]. Furthermore, it has been suggested that hyperinsulinemia might contribute to altered ovarian morphology and arrested follicular development [14, 66]. Impaired insulin signaling, which might be a byproduct of hyperinsulinemia, has also been associated with abnormal LH release, both during tonic secretion [64] and during the preovulatory surge [76].

A. Impaired Positive Feedback on LH Surge

A previous study by Sagae *et al.* [63] used a cafeteria-style diet and showed an attenuated LH surge on proestrus. Likewise, our HFHS rats showed a significantly decreased LH surge on

proestrus. Additionally, in our study, the HFHS rats exhibited a substantially higher concentration of E2 on the evening of proestrus. Although we do not know whether this high level of E2 resulted from a surge of either greater amplitude or longer duration, the abnormal level of this hormone might be associated with the disrupted LH surge. This might indicate an impairment in the positive feedback loop during the preovulatory surge.

It is well established that positive feedback of E2 is essential for initiation of the preovulatory gonadotropin surge [77–81]. It is thought that kisspeptin neurons in the hypothalamus might act as mediators of steroid feedback, because they have been shown to express estrogen receptor-α and project directly to the gonadotropin-releasing hormone neurons that stimulate gonadotropin release [31, 33, 35, 77, 82–88]. Additionally, kisspeptin expression increases on proestrus in response to E2-positive feedback [32]. However, a study by Quennell *et al.* [89] demonstrated a decrease in kisspeptin expression in diet-induced obese mice, suggesting that disrupted hypothalamic activity might contribute to reproductive malfunction [90]. Reduced sensitivity in kisspeptin neurons to E2-positive feedback has also been associated with a decreased LH surge and reproductive impairment in aging rodents [91]. One explanation of our results, therefore, might be that our HFHS rats were relatively insensitive to the E2-positive feedback that precedes the LH surge. Despite sustained high levels of E2, the HFHS rats exhibited on average, a lower LH surge, suggesting impairment in positive feedback.

It is possible that this reduced sensitivity of LH to E2-positive feedback might be a consequence of impaired P4 signaling in these rats. In the present study, the HFHS rats exhibited a significantly decreased concentration of P4 on proestrus. Previous research has suggested that preovulatory P4 secretion is essential in mediating LH surge-induced ovulation [92]. Studies have shown that the positive feedback exerted by E2 is dependent on subsequent P4 signaling [35–37]. For example, P4 induces and amplifies the LH surge after a priming administration of E2 [92–95]. Moreover, administration of E2 is able to induce the LH surge and subsequent ovulation in rats in persistent estrus, but only after P4 is also administered, suggesting that P4 is critical to the feedback on LH secretion [68, 96, 97]. Several other studies have likewise suggested that anovulatory persistent estrus is associated with impaired P4 signaling [98–100]. An interesting study in cattle also suggested that a lack of P4 signaling during the E2-induced LH surge might result in ovarian cyst formation [101, 102].

Although E2 alone can induce an LH surge in many cases, a normal preovulatory P4 surge might serve to lower the threshold for LH stimulation by E2. The HFHS rats displayed abnormal ovarian morphology, suggesting that the lower level of preovulatory P4 in these rats might have prohibited an LH surge large enough to initiate ovulation. It is possible, therefore, that the altered ovarian morphology of the HFHS rats is a consequence of this hormonal imbalance late in follicular development.

A state of hyperandrogenism has been shown to interfere with P4 feedback [103, 104]. In our study, the HFHS rats exhibited elevated testosterone levels, which might have contributed to altered P4 feedback on LH during positive feedback. However, several studies have found that elevated T is associated with increased basal LH secretion during the negative feedback phase of the estrous cycle [34, 104, 105]. In our study, although we found elevated levels of basal testosterone, we observed decreased levels of LH on diestrus I. This might suggest that other mechanisms, such as changes in leptin or insulin signaling [106–108], are responsible for the alterations in basal hormonal release in our HFHS rats.

B. Bimodal Ovarian Morphology in HFHS Rats

Although a corresponding decrease in CL and increase in cysts might suggest a failure to ovulate in these HFHS rats, a normal number of GFs might indicate that the disruption in ovulation occurs late in the cycle or that HFHS rats resist follicular atresia to a greater degree until ovulation. In evaluating the ovaries of the HFHS rats, we observed a bimodal distribution in ovarian morphology. One group of rats displayed a relatively small number of cysts with overall normal ovarian morphology, and a second group had an excessive number of cysts

that corresponded with completely altered ovarian morphology. These highly cystic rats lacked normal ovarian structures such as CL and antral follicles. We also found a trend toward a greater number of GFs in the HFHS group ($P = 0.07$), suggesting that despite a high GF count, the HFHS rats failed to ovulate. Polycystic ovaries have been associated with a pool of growing follicles two to three times greater than in normal ovaries [109]. It has been suggested that T might be responsible for this excessive initial follicle recruitment [110, 111]. Along with T, changes in AMH levels have also been implicated in follicular growth disruption and in obesity-related infertility [112–114]. However, we found no relevant difference in AMH levels between the two groups (data not shown). It is possible that more follicles were selected at early stages for maturation, as a consequence of the high circulating levels of T seen in the HFHS rats. A subset of these follicles might have reached full maturation to the Graafian stage; however, in light of a lower LH surge and a potentially disrupted positive feedback loop, ovulation failed to occur [115]. This might explain the appearance of a high number of GF in the HFHS rats but the few subsequent CL.

Recent studies have further focused on androgen receptors, especially in hyperandrogenized animal models of PCOS [116]. Androgen receptors are highly concentrated in granulosa cells and are primarily localized in preantral and antral follicles of rodents and primates [117–119]. Through androgen receptors, androgens have been shown to stimulate growth of theca interna and granulosa cells and thus are implicated as critical factors in the early process of follicle maturation [120, 121]. It is likely that high circulating levels of T increased the initial pool of selectable follicles and might have led to an early proliferation of mature follicle development. However, the lack of an LH surge presumably leads to the progressive development of cysts that dominate the ovary.

C. HFHS Diet Alters Steroidogenesis Pathway

Although we discovered alterations in the steroid hormones that contribute to the preovulatory gonadotropin surge, we also investigated the contribution of basal steroid hormone levels to ovarian morphology. The ovaries serve as the site of steroid hormone production, and alterations in steroidogenesis have been implicated in the development of reproductive dysfunction [122–124]. After examining relevant ratios of steroid hormones, we found that the HFHS rats as a group exhibited, on average, a substantially increased T/E2 ratio. Because E2 follows T in the steroidogenesis pathway, this might suggest either a decrease in the conversion rate of T to E2 or an increase in T production in the HFHS rats. However, we found no difference in E2 levels between the two groups, suggesting that this ratio is driven by increased androgen production. The elevated serum T levels seen in the HFHS group support this claim. Although we do not know whether this resulted from reduced aromatase activity in the granulosa cells that convert androgens to E2 or an increase in theca cell androgen production, studies have shown that increased thecal cell androgen production is a hallmark of reproductive disorders [123, 125]. Thus, to determine whether T levels correlated with altered ovarian morphology in our study, we then correlated the T levels with the cyst counts. However, T was not the strongest predictor of cyst formation in individual HFHS rats. Instead, we found a positive exponential relationship between an individual rat's P4/T ratio and cyst counts. In rodents, testosterone is a derivative of P4 in the steroidogenesis pathway occurring in theca cells [122]. In a normal ovary after ovulation, theca cells favor P4 production and switch from androgen-producing to P4-producing pathways [126]. In our study, the rats with high cyst counts had remarkably low P4/T ratios, suggesting an impairment in this switch mechanism. These correlations further suggest that individual HFHS rats with low P4/T ratios might be more susceptible to cyst-like formation and that a high cyst count might contribute to disruption in the steroidogenesis pathway and in estrous cyclicity. Moreover, the other steroid hormone ratios in these animals did not accurately predict cyst or CL formation, suggesting that the initial P4/T conversion is a critical step in an individual animal's development of abnormal ovarian morphology. Other studies have suggested that increased activity of the enzyme responsible for the P4/testosterone conversion in rats, CYP17

(also called 17-α-hydroxylase), might contribute to the development of reproductive disorders such as PCOS [124, 127, 128]. Thus, it might be that an increase in steroidogenic enzymatic activity contributes to an increased conversion of P4 to T seen among individual rats in our study. The steroidogenesis pathway elucidated in our rats differs from that in humans [129]; however, this increased rate of conversion might serve as an indicator of cyst-like development per individual animal in our study.

4. Conclusion

In the present study, we used a HFHS diet in a rodent model of diet-induced obesity. Our HFHS model has provided a unique opportunity to determine progressive changes in hormone levels that might underlie the development of an abnormal reproductive phenotype. The HFHS diet resulted in an impaired preovulatory hormonal surge and altered basal hormone levels. An imbalance in basal steroid hormones further correlated strongly with ovarian cyst formation. Thus, our model offers a method to examine the morphological and physiological changes that might contribute to the disruption of reproductive cycle. Our study ultimately contributes to the understanding of the direct role that diet plays in the generation of reproductive dysfunction.

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