UNIVERSITY OF CALIFORNIA SAN DIEGO

Exploring Insulin Resistance and the Protective Effect of Fiber in Polycystic Ovary Syndrome

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requirements for the degree Master of Science

in

Biology

by

Annie Chen

Committee in charge:

Professor Varykina Thackray, Chair
Professor Heidi Cook-Andersen, Co-chair
Professor James Cooke

2021

The thesis of Annie Chen is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

2021

DEDICATION

I'd like to dedicate my thesis to:

My mom, dad, and younger brother Andrew

Ethan

Joy

Joanna, Phillip, and Michelle

The Thackray lab

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LIST OF ABBREVIATIONS

ARC	arcuate nucleus
AVPV	anteroventral periventricular nucleus
BMI	body mass index
Con	control diet
CRP	c-reactive protein
DHT	dihydrotestosterone
E2	17β-estradiol
αERKO	estrogen receptor α knock out
FBG	fasting blood glucose
FBI	fasting blood insulin
Fib	5% benefiber diet
FSH	follicle-stimulating hormone
GnRH	gonadotropin-releasing hormone
HFD	high-fat diet
HPG	hypothalamic-pituitary-gonadal axis
ITT	insulin tolerance test
Kiss1	kisspeptin
LET	letrozole
LH	luteinizing hormone
OVX	ovariectomy
P	placebo
P4	progesterone
PCOS	polycystic ovary syndrome
SHAM	sham-operated
SCFA	short chain fatty acid

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ABSTRACT OF THE THESIS

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Annie Chen

Master of Science in Biology

University of California San Diego, 2021

Professor Varykina Thackray, Chair
Professor Heidi Cook-Andersen, Co-chair

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder among women of reproductive age. The etiology of this disease is poorly understood, and there is also no known cure. PCOS features both reproductive and metabolic dysfunction and can have devastating and long-term health consequences. Further research is needed to better understand this disease and to develop new therapeutic approaches for treating this disorder.

Because many women who have PCOS also have some degree of insulin resistance, we investigated possible contributors to insulin resistance in a letrozole-induced PCOS-like mouse model. We found that hyperandrogenism from dihydrotestosterone (DHT) treatment or estrogen deficiency from ovariectomy (OVX) was not sufficient to induce insulin resistance in female mice. However, mice that received both DHT and OVX became insulin resistant, suggesting that a combination of elevated androgen levels and lowered estrogen levels may be necessary for the development of insulin resistance in PCOS.

The gut microbiome has been implicated in various diseases such as metabolic syndrome and PCOS. More specifically, multiple studies have demonstrated that changes in the gut microbiome are associated with PCOS in humans and rodent models, and studies in mice have demonstrated that altering the gut microbiome can alleviate PCOS pathology. We investigated whether dietary supplementation with a prebiotic, resistant fiber was protective against the development of PCOS reproductive and metabolic phenotypes in a letrozole-induced PCOS-like mouse model. We found that 5% resistant fiber supplementation was not sufficient to protect against development of PCOS phenotypes, suggesting that the effect of supplementation with a greater percentage of dietary fiber or different types of dietary fiber should be explored further.

CHAPTER I: INTRODUCTION

The Hypothalamic-Pituitary-Gonadal Axis

Comprised of the hypothalamus, pituitary gland, and gonads, the hypothalamic-pituitary-gonadal (HPG) axis is the major neuroendocrine system that regulates sexual development and reproduction[1–3]. In the hypothalamus, kisspeptin (Kiss1) released by Kiss1 neurons within the arcuate (ARC) nucleus binds to Kiss1 receptors located on gonadotropin releasing hormone (GnRH) neurons and stimulates pulsatile release of GnRH into the portal veins[2–5] **(Figure 1)**. GnRH causes gonadotrope cells of the anterior pituitary to produce and release follicle stimulating hormone (FSH) and luteinizing hormone (LH) into the bloodstream, which ultimately triggers production of sex steroids, such as testosterone (T) and 17β-estradiol (E2), and gametogenesis in the gonads[6–9] **(Figure 1)**. Both androgens and estrogens can negatively feedback on the hypothalamus and pituitary to regulate sex steroid concentrations[10–13] **(Figure 1)**. However, only estrogens participate in a positive feedback loop with the Kiss1 neurons in the anteroventral periventricular (AVPV) nucleus to produce an LH surge that triggers ovulation in females[11,14,15] **(Figure 1)**.

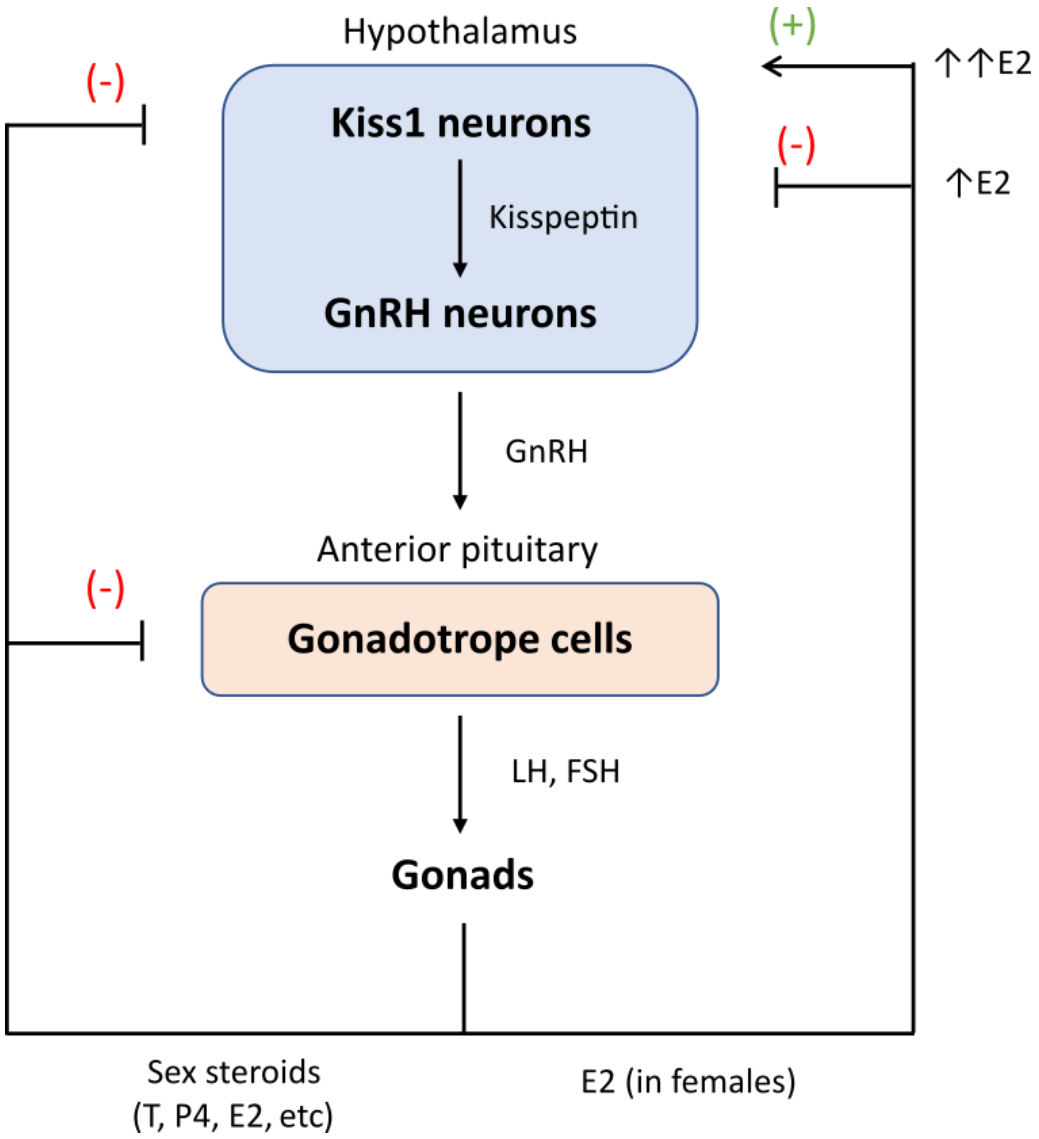


Figure 1: The hypothalamic-pituitary-gonadal (HPG) axis is a major regulator of reproduction.

Kisspeptin (Kiss1) neurons in the hypothalamus release kisspeptin, which stimulates the pulsatile release of gonadotropin-releasing hormone (GnRH) from GnRH neurons. GnRH then triggers release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) from gonadotrope cells of the anterior pituitary. LH and FSH increase gametogenesis along with the production of sex steroids such as testosterone (T) and estradiol (E2), which negatively feedback onto the hypothalamus and anterior pituitary. In females, high levels of E2 participate in positive feedback in the anteroventral ventricular nucleus (AVPV) to trigger an LH surge and consequently ovulation.

Estrogens

Comprised of estrone, estradiol, and estriol, estrogens are sex steroids that regulate reproduction and sexual development. The gonads and other tissues, such as adipose and brain, utilize an enzyme called aromatase to convert various androgens such as androstenedione and testosterone into estrogen[16–18] **(Figure 2A)**. In pre-menopausal women, estradiol (E2) is the primary estrogen in circulation and the most potent. Estrogen drives the development of female secondary sex characteristics and stimulates growth of the endometrial lining in preparation for pregnancy[18–21]. Furthermore, high levels of estrogen can trigger both the luteinization of granulosa cells, which initiates progesterone (P4) production, and an LH surge to induce ovulation[11,14,15].

While the primary role of estrogen is in regulating reproduction, clinical research into estrogen deficiency has highlighted the importance of this sex steroid in metabolism, bone maintenance, and even behavior[22–24]. Estrogen deficiency can be caused by hypothalamic-pituitary insufficiency, as seen in hyperprolactinemia, anorexia nervosa, and Kallmann Syndrome[25,26]. It can also be caused by surgery, chemotherapy, GnRH agonist treatment, or other conditions that result in ovarian insufficiency[27–29]. Post-menopausal women, who have declining levels of estrogen, tend to experience weight gain and changes in mood and behavior, and are at higher risk for cardiovascular disease[30–33]. Estrogen also has a protective effect on bone density by preventing resorption, and prolonged hypoestrogenism in young women and post-menopausal women is associated with an increased risk of developing pathological fractures due to osteoporosis and osteopenia[34–37]. Altogether, the problems that arise from insufficient estrogen levels demonstrate how estrogen is crucial for both reproductive function and overall health.

Androgens

Androgens are the other major category of sex steroids aside from estrogens. Among the many different androgens that are endogenous to the human body, testosterone (T) is the primary circulating androgen in both males and females, but it is not the most potent. T can be converted into a more potent and nonaromatizable androgen known as dihydrotestosterone (DHT) by the enzyme 5α-reductase[38–40] **(Figure 2A)**. In males, androgens are produced at high levels by the testes and drive important processes involved in fertility such as sperm production and the development of male secondary sex characteristics[40–44]. Females also produce androgens in the ovaries and adrenal glands but convert the majority into estrogens via aromatization. Aside from male reproduction, androgens are also involved in regulating fat deposition[45,46], muscle mass[47–49], and brain development in both males and females[50–52]. However, because androgen levels tend to be significantly lower in females compared to males, excess androgen levels can be pathological for women and can manifest as disorders such as polycystic ovary syndrome.

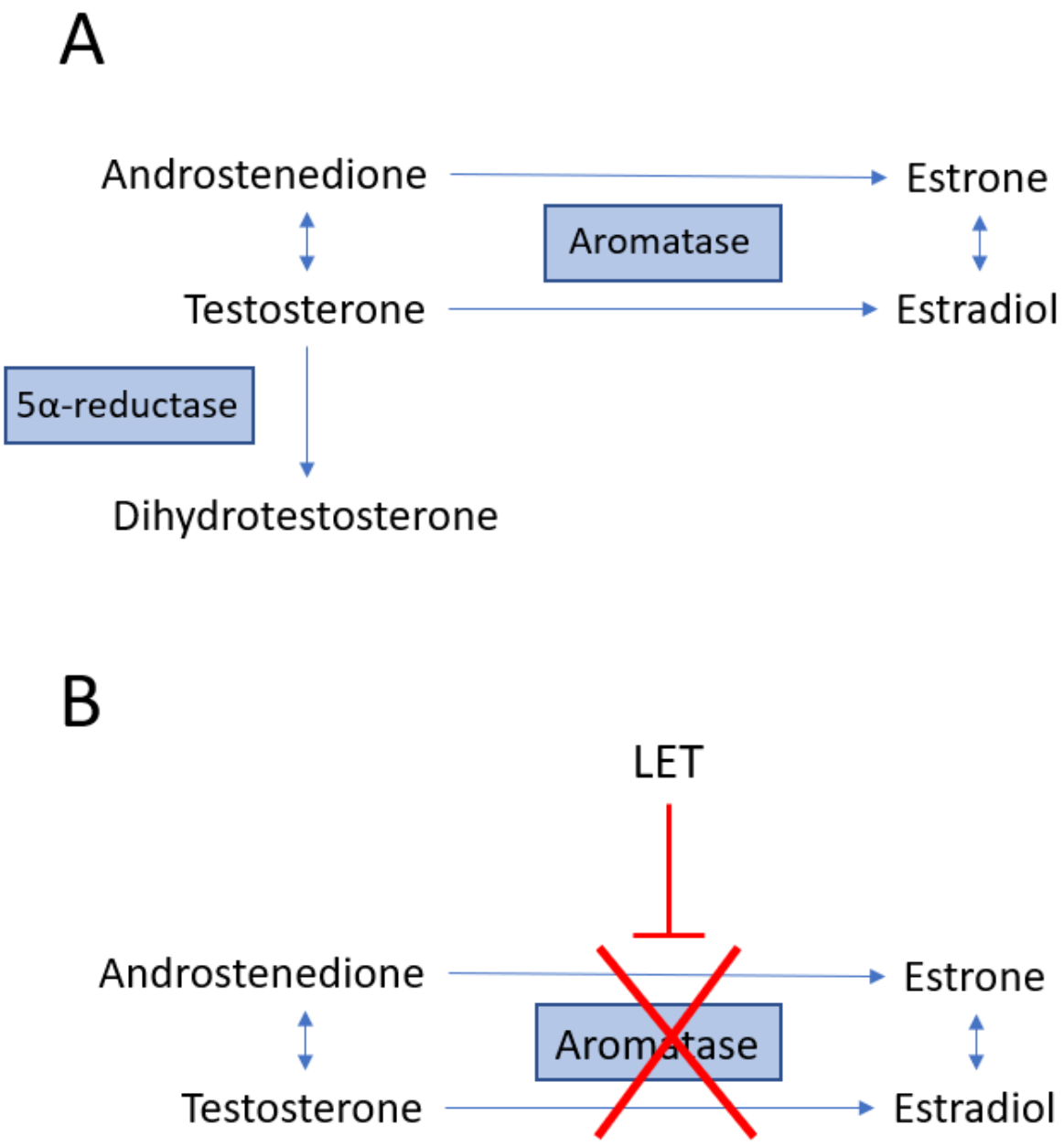


Figure 2: By inhibiting the aromatase enzyme, letrozole prevents the conversion of androgens into estrogens.

(Panel A) The ovaries, adipose tissue, and other tissues contain an enzyme known as aromatase, which converts androgens such as testosterone into estrogens such as estradiol. Testosterone can also be converted into dihydrotestosterone through 5α-reductase**. (Panel B)** Letrozole (LET) is a nonsteroidal aromatase inhibitor. Upon administration, LET prevents the aromatization of androgens into estrogens, causing a buildup of androgens and lowered estrogen levels.

Polycystic Ovary Syndrome

Polycystic ovary syndrome (PCOS) is a common endocrine disorder affecting approximately 10-15% of reproductive-age women[53]. In order to be diagnosed, at least 2 out of 3 Rotterdam Consensus criteria must be met: hyperandrogenism, oligomenorrhea or amenorrhea, and polycystic ovaries[54]. Reproductive dysfunction is a hallmark of PCOS, as patients are at greater risk for infertility due to anovulation, miscarriage, and pregnancy complications[55,56]. An increased LH to FSH ratio is also characteristic of PCOS[57]. In addition to reproductive problems, PCOS is also associated with a strong metabolic phenotype including obesity, insulin resistance, dyslipidemia, and hypertension, all of which increase the risk of developing cardiovascular disease, Type 2 Diabetes, and non-alcoholic fatty liver disease[58–62].

Despite such high prevalence, the etiology of PCOS has yet to be determined, but twin studies have demonstrated that PCOS is likely caused by a combination of both genetic factors and environmental factors, such as prenatal androgen exposure[63–65]. Genome-wide association studies have also identified multiple single-nucleotide polymorphisms that are associated with PCOS, indicating that this is a polygenic disorder[66,67]. Additionally, the majority of hyperandrogenic women with PCOS have some degree of insulin resistance that is independent of weight or BMI, suggesting that insulin resistance may play an important role in PCOS pathophysiology[68–70]. Because the causes of PCOS are still unknown, there is no known cure for this disease. Common treatment options for symptom management include hormonal oral contraceptives to decrease androgen production in women not seeking pregnancy and dietary or lifestyle changes to improve metabolic health[55,60].

Dihydrotestosterone, Letrozole, and Ovariectomized Mouse Models

Because excess androgen levels are viewed as a central component of PCOS pathology, hyperandrogenic rodent models are commonly used to study PCOS[71–73]. In particular, the potent androgen DHT cannot be aromatized into estrogen and is often used to induce hyperandrogenism instead of testosterone, which would have the confounding variable of increased estrogen. Treatment with excess DHT causes reproductive dysfunction, such as hyperandrogenism, acyclicity, and polycystic ovaries. However, DHT mice do not have elevated LH levels or increased ovarian weight that are characteristic in women with PCOS[57,74–77].

The severity of the metabolic phenotype, such as weight gain and adiposity, depends on the dosage of DHT used and the time frame of the study[74,77–79]. Given the high comorbidity between PCOS and insulin resistance, simulating insulin resistance in a PCOS mouse model is crucial to understanding this disease[68,69]. Some studies reported fasting blood glucose (FBG) or results from glucose tolerance tests as evidence of potential insulin resistance in DHT-treated mice, but these parameters are more reflective of glucose homeostasis and do not appropriately assess insulin resistance. It is also noteworthy that most metabolic phenotypes observed in DHT mouse models were the result of prolonged (3 month) treatment of DHT, and the effects of short-term DHT treatment are not well characterized[74,77,78,80]. Overall, the DHT mouse model simulates the reproductive phenotypes of PCOS but lacks a strong metabolic phenotype, especially with regards to insulin resistance.

In contrast, the pubertal letrozole (LET) mouse model recapitulates both reproductive and metabolic phenotypes of PCOS[81]. In this model, mice are treated with letrozole, a non-steroidal aromatase inhibitor **(Figure 2B)**. LET treatment prevents the conversion of androgens into estrogens, causing the build-up of excess androgens while decreasing estrogen production in

pubertal mice **(Figure 2B)**. With short-term treatment from puberty to adulthood, LET mice exhibit reproductive dysfunction such as hyperandrogenemia, polycystic ovaries, elevated LH levels, and acyclicity[81,82]. PCOS metabolic phenotypes are also robust in this model, as LET mice have substantial weight gain, abdominal adiposity, dyslipidemia, and elevated fasting blood insulin (FBI)[81]. Most importantly, LET treatment results in insulin resistance that does not appear to be recapitulated in the DHT model[83]. When comparing the DHT and LET mouse models, the main difference between the models is that DHT treatment results only in hyperandrogenism whereas LET treatment results in both hyperandrogenism and estrogen deficiency, suggesting that decreased estrogen levels may play a role in the development of insulin resistance.

The ovaries are the main site of estrogen production. Consequently, removal of the ovaries results in low estrogen levels, creating an ovariectomized (OVX) mouse model that can be used to study the effects of estrogen deficiency[84,85]. OVX mice have weight gain, abdominal adiposity, and elevated FBG and FBI and thus share many metabolic similarities with LET mice, but OVX mice do not develop insulin resistance after 5 weeks[84,85]. Results from DHT and OVX mouse models demonstrate that hyperandrogenism or estrogen deficiency alone may not be sufficient to induce insulin resistance. Therefore, an OVX + DHT mouse model was utilized to investigate whether a combination of hyperandrogenism and estrogen deficiency would be sufficient to induce insulin resistance.

CHAPTER I: MATERIALS AND METHODS

Mouse Models

Three-week-old C57BL/6N female mice were purchased from Envigo and housed in a vivarium with an automatic 12 h light:12 h darkness cycle (light period: 6:00AM to 6:00PM). Mice were given ad libitum access to water and food (Teklad Global 18% Protein Extruded Diet; Envigo). Mice were weighed each week. All animal procedures in this study were approved by the University of California, San Diego Institutional Animal Care and Use Committee (Protocol S14011).

DHT Mouse Models:

At four weeks of age, all mice were implanted subcutaneously with either a DHT (Steraloids) or empty (placebo; P) implant made in-house.

For the 4-mm DHT model, an 8-mm Silastic implant (i.d., 1 mm; o.d., 2.15 mm; Dow Corning; 1118915D) containing 2 mg of DHT within a 4-mm space was used. This created two experimental groups (P or DHT) with n=8/group.

For the 10-mm DHT model, a 14-mm Silastic implant (i.d., 1.47 mm; o.d., 1.96 mm; Dow Corning; 508-006) containing 8.8 mg of DHT within a 10-mm space was used. This created two experimental groups (P or DHT) with n=10/group.

OVX + DHT Mouse Model:

At four weeks of age, all mice underwent either a sham (SHAM) surgery or OVX. Additionally, mice were implanted subcutaneously with either a DHT or empty implant, as

described in the 4-mm DHT model above. Surgery and pellet combinations created four experimental groups: SHAM + P, OVX + P, SHAM + DHT, and OVX + DHT (n=10/group).

Estrous Cycle Assessment

Estrous cycles of the mice were determined during weeks 4-5 of treatment by light microscopy examination of vaginal cytology for seven days, as previously described[86]. Diestrus consisted predominantly of leukocytes; proestrus of nucleated epithelial cells; estrus of cornified epithelial cells; metestrus of a combination of cornified and nucleated epithelial cells along with leukocytes.

Insulin Tolerance Test

At 5 weeks of treatment, mice were fasted for 5 hours. A handheld glucometer (One Touch UltraMini; LifeScan, Inc.) was used to measure FBG at all timepoints. Tail blood was collected and FBG was measured at time point 0 before administration of an intraperitoneal injection of insulin (0.75 U/kg in sterile saline; Humulin R U-100). Blood glucose was measured at 15, 30, 45, 60, 90, and 120 minutes post insulin injection. Collected tail blood was used to measure fasting insulin levels in serum via ELISAat the UC Davis Mouse Metabolic Phenotyping Center.

Tissue Collection

5 weeks post implantation, mice were euthanized with 2.5% isoflurane. Terminal blood was collected from the inferior vena cava for hormone assays. Parametrial fat pads were dissected and weighed.

Hormone Assays

Terminal blood collected at week 5 was used to assess hormone levels. Radioimmunoassay was used to measure LH levels (range 0.02-75 ng/mL) at the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core Facility. DHT levels (range 0.093-750 ng/mL) were measured by extraction/chromatography radioimmunoassay at the Oregon National Primate Research Center Endocrine Technologies Core.

Statistical Analyses

The statistical package JMP 15 was used to analyze differences among groups. Data residuals were checked for normality and one-way ANOVA followed by post hoc Tukey-Kramer honestly significant difference test (4 groups) or student t-test (2 groups) was performed. If residuals were not normal, data underwent Box-Cox transformation, and non-parametric tests were performed if transformation did not result in normality. Statistical significance ($P<0.05$) was indicated by different letters or an asterisk.

Fecal Sample Collection and DNA Isolation

Fecal samples were collected from all mice before treatment and once per week during the 5 weeks of the experiment. Fecal samples were frozen and stored at -80ºC. Bacterial DNA extracted from the samples using the PowerSoil DNA Isolation kit (Qiagen) were amplified via polymerase chain reaction using primers 515F and 806R, as previously described[87]. Amplicon sequence libraries were prepared at the Scripps Research Institute Next Generation Sequencing Core Facility and sequenced on an Illumina MiSeq.

RESULTS

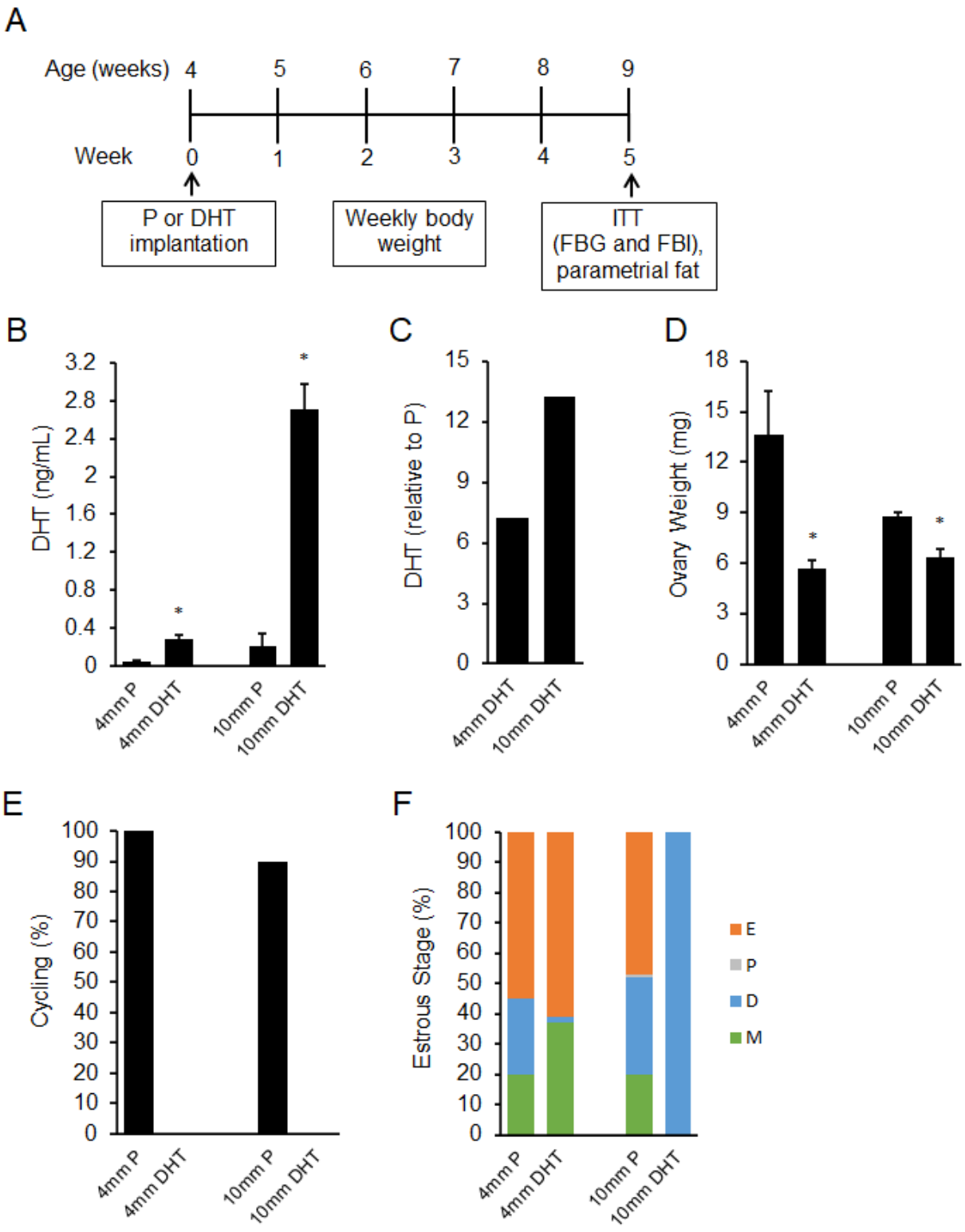
CHAPTER I: Hyperandrogenemia combined with estrogen deficiency is sufficient to induce insulin resistance

Elevated dihydrotestosterone levels induced acyclicity in mice

Several hyperandrogenic mouse models have been used to model PCOS-like phenotypes, including 3 months of treatment with a 4mm implant containing 2mg of DHT that induces a modest increase in androgens in adult females[79] and a 10mm implant containing 8.8mg of DHT that mimics male-like levels of androgens[74]. Since it was unclear whether short-term DHT treatment induces reproductive and metabolic phenotypes, 4-week-old female mice were treated with either P or DHT for five weeks (**Figure 3A**). 4mm DHT treatment resulted in a 7-fold increase in serum DHT levels, whereas 10mm DHT induced a 13-fold increase (**Figure 3B** and **Figure 3C**). When ovaries were weighed at the end of the experiment, ovaries of mice treated with 4mm or 10 mm DHT were lighter than those of P mice (**Figure 3D**). Vaginal cytology revealed that mice treated with DHT were acyclic and stuck in various stages of the estrous cycle (**Figure 3E** and **Figure 3F**).

Figure 3: Dihydrotestosterone-treated mice developed reproductive dysfunction.

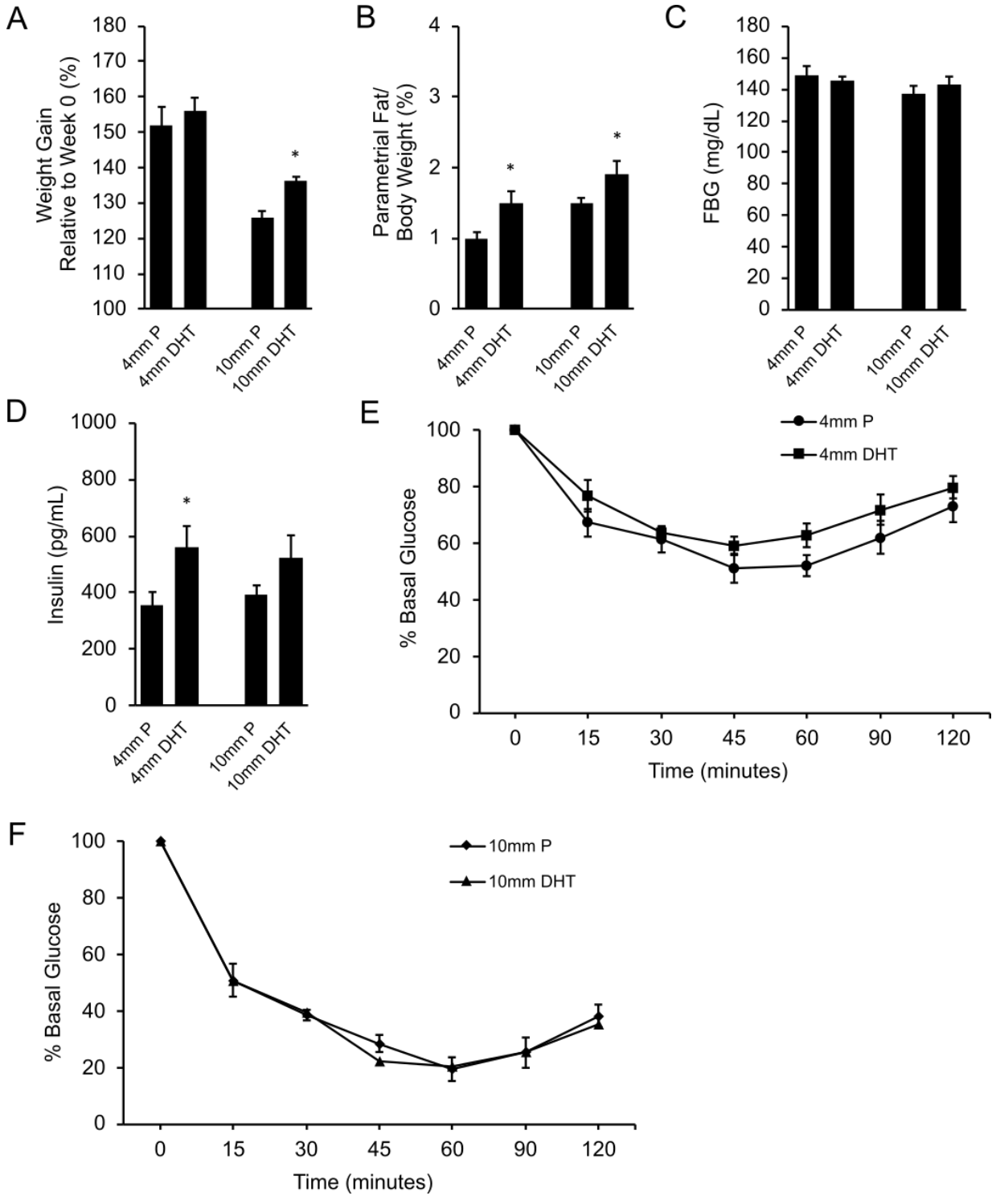
(Panel A) Schematic of experimental procedures: Mice were given either a placebo (P) or dihydrotestosterone (DHT) implant at 4 weeks of age (n = 8/group for 4mm, n = 10/group for 10mm). Weekly weight assessments, an insulin tolerance test (ITT), and measurement of fasting blood glucose (FBG), fasting blood insulin (FBI), and parametrial fat weight were performed. **(Panel B)** Mice treated with DHT had significantly higher levels of serum DHT compared to mice treated with P. **(Panel C)** Treatment with 4mm DHT led to a 7-fold increase in serum DHT compared to P, whereas treatment with 10mm DHT led to a 13-fold increase. **(Panel D)** Mice treated with 4mm or 10mm DHT had ovaries that weighed significantly less than P ovaries. **(Panel E)** Mice treated with 4mm or 10mm DHT did not have regular estrous cycles compared to P mice. **(Panel F)** 4mm DHT mice were stuck in either the metestrus or estrus stage, while 10mm DHT mice were stuck in the diestrus stage. Standard error of the mean is represented by graph error bars. Student t-test comparing each DHT group to its respective P group was used; $p < 0.05$.


A
Age (weeks) 4 5 6 7 8 9
Week 0 1 2 3 4 5
P or DHT implantation
Weekly body weight
ITT (FBG and FBI), parametrial fat
B
DHT (ng/mL)
4mm P
4mm DHT
10mm P
10mm DHT
C
DHT (relative to P)
4mm DHT
10mm DHT
D
Ovary Weight (mg)
4mm P
4mm DHT
10mm P
10mm DHT
E
Cycling (%)
4mm P
4mm DHT
10mm P
10mm DHT
F
Estrous Stage (%)
E
P
D
M
4mm P
4mm DHT
10mm P
10mm DHT

Dihydrotestosterone-treated mice had a mild metabolic phenotype but no irregularities in glucose homeostasis

After five weeks of treatment, only 10mm DHT mice gained body weight relative to P mice (**Figure 4A**). However, both 4mm and 10mm DHT mice displayed a significant increase in abdominal adiposity (**Figure 4B**). While FBG levels were found to be similar among 4 mm, 10mm DHT and P mice, 4mm DHT mice had elevated insulin levels compared to P (**Figure 4C** and **Figure 4D**). DHT-treated mice did not show differences in their response to insulin compared to P during an ITT, indicating that they were not insulin resistant (**Figure 4E** and **Figure 4F**).

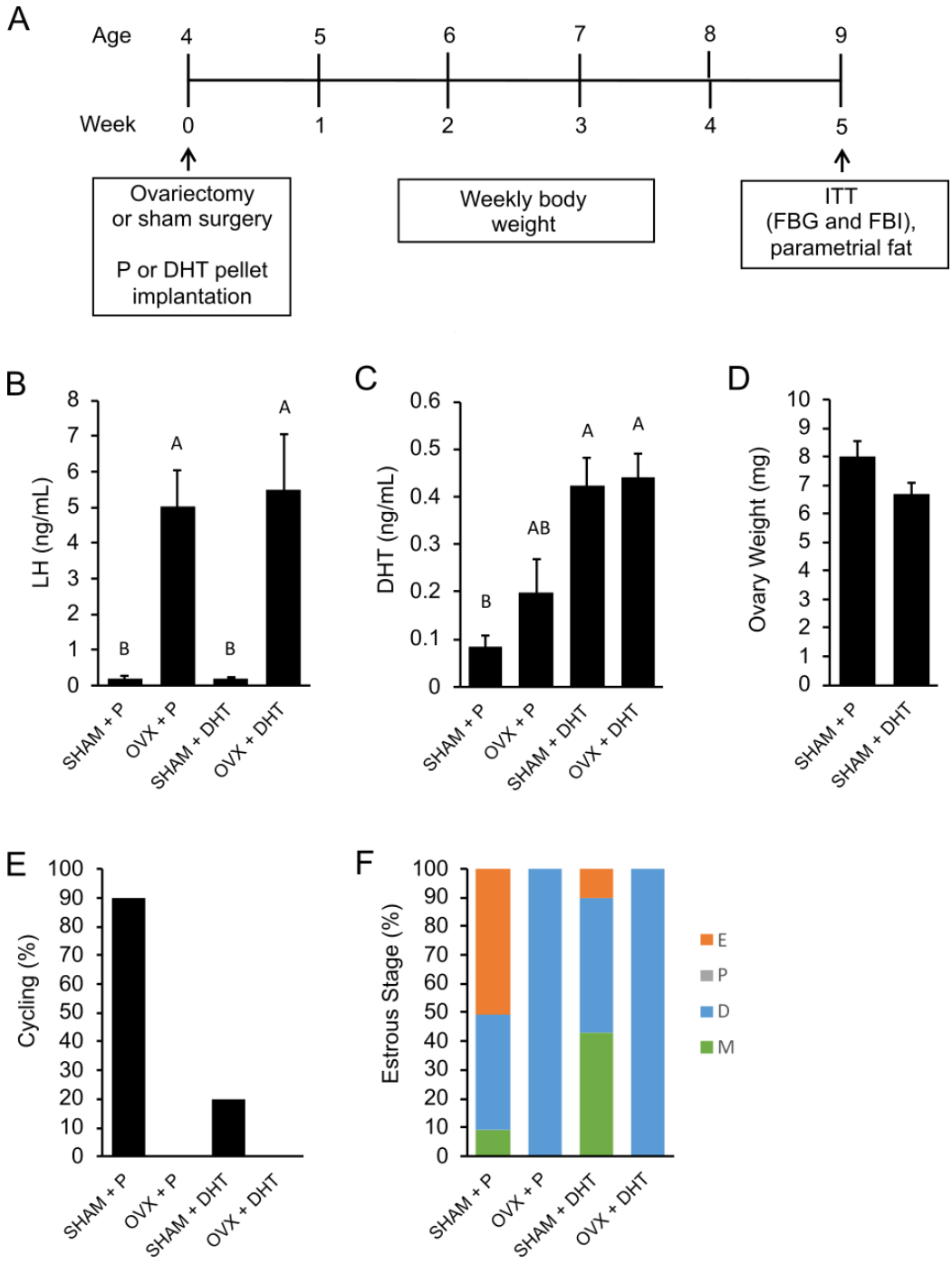
Figure 4: Treatment with 4mm or 10mm dihydrotestosterone (DHT) resulted in a mild metabolic phenotype. (Panel A) Treatment with 10mm DHT caused significantly more weight gain compared to placebo (P) or treatment with 4mm DHT after 5 weeks of treatment. **(Panel B)** Treatment with 4mm and 10mm DHT resulted in increased parametrial fat relative to body weight compared to P. **(Panel C)** Compared to P mice, DHT mice showed no significant differences in fasting blood glucose (FBG). **(Panel D)** Treatment with 4mm DHT resulted in elevated insulin levels that were not seen with 10mm DHT. **(Panel E-F)** Insulin tolerance was similar between DHT and P mice. Standard error of the mean is represented by graph error bars. Student t-test comparing each DHT group to its respective P group was used; $p < 0.05$.


A
Weight Gain
Relative to Week 0 (%)
180
170
160
150
140
130
120
110
100
4mm P
4mm DHT
10mm P
10mm DHT
*
B
Parametrial Fat/
Body Weight (%)
4
3
2
1
0
*
*
C
FBG (mg/dL)
180
160
140
120
100
80
60
40
20
0
D
Insulin (pg/mL)
1000
800
600
400
200
0
*
E
% Basal Glucose
100
80
60
40
20
0
4mm P
4mm DHT
0
15
30
45
60
90
120
Time (minutes)
F
% Basal Glucose
10mm P
10mm DHT
Time (minutes)

Mice given an ovariectomy and dihydrotestosterone treatment lacked regular estrous cyclicity

Since OVX or DHT-treated mice lacked a PCOS-like metabolic phenotype[85], we investigated whether the combination of low estrogen and elevated androgens were required to develop metabolic dysregulation. In this experiment, four-week-old mice were either OVX or SHAM and treated with either P or DHT for five weeks (**Figure 5A**). Because removal of the ovaries reduces negative feedback from estrogen, OVX + DHT mice had elevated levels of serum LH similar to OVX + P mice (**Figure 5B**). OVX + DHT mice also had elevated serum levels of DHT that were similar to SHAM + DHT (**Figure 5C**). No significant difference was found in ovary weight between the two SHAM groups treated with either P or DHT (**Figure 5D**). Similarly, to OVX or DHT mice, mice with OVX + DHT lacked regular estrous cycles and were stuck in the diestrus stage (**Figure 5E** and **Figure 5F**).

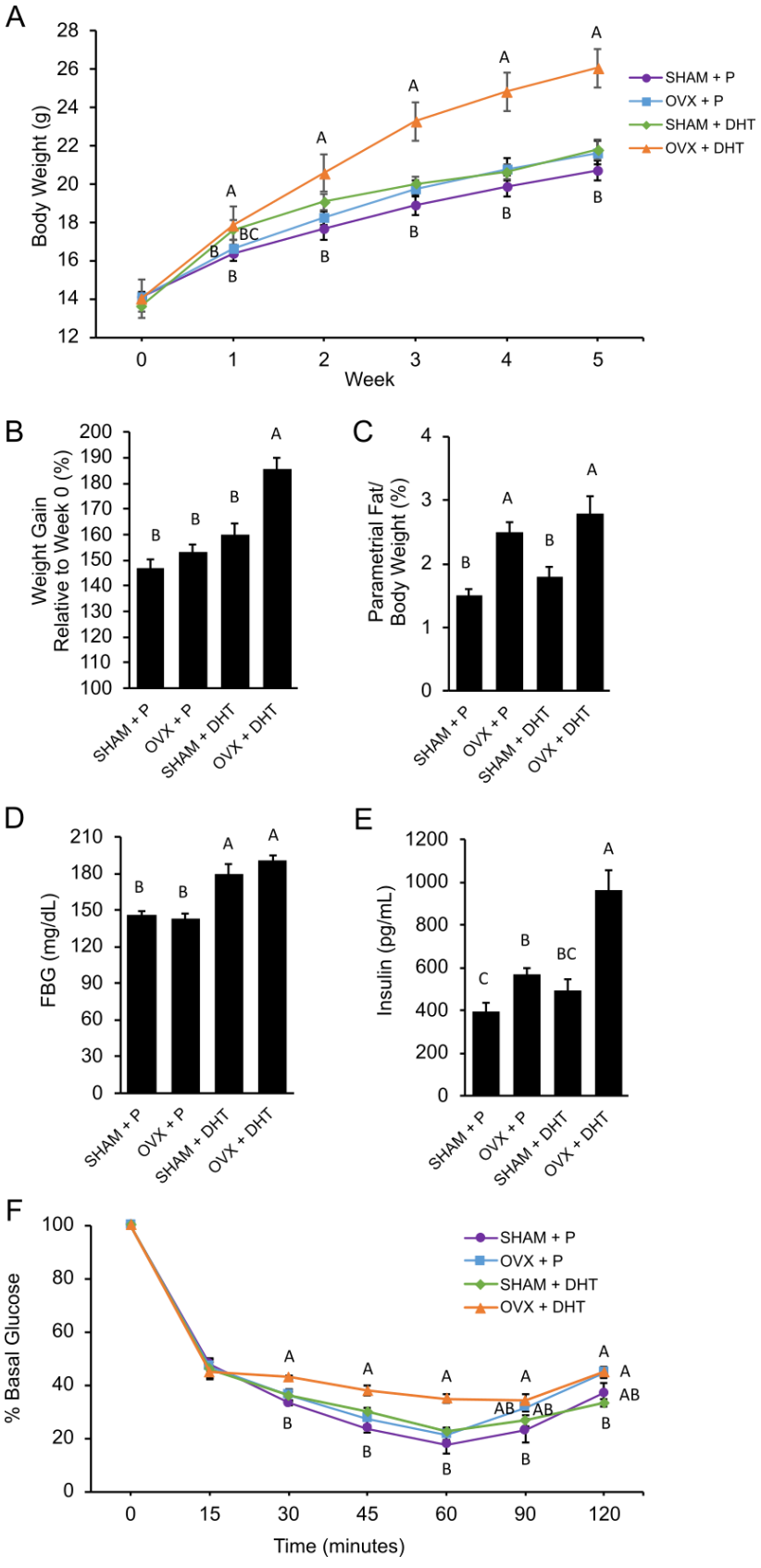
Figure 5: Mice with ovariectomy (OVX) and dihydrotestosterone (DHT) treatment developed acyclicity. (Panel A) Schematic of experimental procedures: Mice were given either a placebo (P) or dihydrotestosterone (DHT) pellet implant, along with either an ovariectomy (OVX) or sham surgery (SHAM) at 4 weeks of age (n = 10 per group). Weekly weight assessments, an insulin tolerance test (ITT), measurement of fasting blood glucose (FBG), fasting blood insulin (FBI), and parametrial fat weight were performed. **(Panel B)** Like OVX + P mice, OVX + DHT mice demonstrated elevated serum luteinizing hormone (LH) levels compared to SHAM + P and SHAM + DHT mice, indicating a lack of estrogen negative feedback. **(Panel C)** Similar to SHAM + DHT mice, OVX + DHT mice had significantly higher serum DHT levels compared to SHAM + P. **(Panel D)** There was no difference in ovary weight between SHAM + P and SHAM + DHT mice. **(Panel E-F)** All OVX + DHT mice were acyclic and were stuck in the diestrus stage. Standard error of the mean is represented by graph error bars. Different letters signify significant differences among groups using a one-way ANOVA followed by post-hoc comparisons with the Tukey-Kramer honestly significant difference test ($p < 0.05$).


A
Age
4
5
6
7
8
9
Week
0
1
2
3
4
5
Ovariectomy or sham surgery
P or DHT pellet implantation
Weekly body weight
ITT (FBG and FBI), parametrial fat
B
LH (ng/mL)
C
DHT (ng/mL)
D
Ovary Weight (mg)
SHAM + P
OVX + P
SHAM + DHT
OVX + DHT
E
Cycling (%)
F
Estrous Stage (%)
E
P
D
M

Ovariectomized and dihydrotestosterone-treated mice displayed a strong PCOS-like metabolic phenotype that included insulin resistance

After one week post-surgery, OVX + DHT mice gained significantly more weight than the other three groups, maintaining this trend until the end of the experiment (**Figure 6A** and **Figure 6B**). In addition to weight gain, OVX + DHT mice also had greater abdominal adiposity reflected by an increase in parametrial fat relative to body weight that was similar to OVX + P mice (**Figure 6C**). As for glucose homeostasis, OVX + DHT mice had elevated FBG and were severely hyperinsulinemic (**Figure 6D** and **Figure 6E**). OVX + DHT mice also displayed insulin intolerance during an ITT compared to the other three groups that did not display insulin resistance (**Figure 6F**).

Figure 6: Ovariectomy (OVX) and dihydrotestosterone (DHT) treatment resulted in a more severe metabolic phenotype compared to OVX or DHT treatment alone. (Panel A-B) OVX + DHT mice gained significantly more weight compared to the other three groups. **(Panel C)** Similar to OVX + P mice, OVX + DHT mice had greater parametrial fat relative to body weight compared to SHAM + P and SHAM + DHT. **(Panel D)** Similar to SHAM + DHT mice, OVX + DHT mice had significantly higher fasting blood glucose (FBG). **(Panel E)** OVX + DHT mice had significantly elevated levels of fasting insulin compared to the other three groups. **(Panel F)** Among the four groups, only OVX + DHT mice displayed insulin intolerance. Standard error of the mean is represented by graph error bars. Different letters signify significant differences among groups using a one-way ANOVA followed by post-hoc comparisons with the Tukey-Kramer honestly significant difference test ($p < 0.05$).


A
Body Weight (g)
Week
SHAM + P
OVX + P
SHAM + DHT
OVX + DHT
B
Weight Gain Relative to Week 0 (%)
C
Parametrial Fat/ Body Weight (%)
D
FBG (mg/dL)
E
Insulin (pg/mL)
F
% Basal Glucose
Time (minutes)

Mice given both an ovariectomy and dihydrotestosterone treatment had a reproductive phenotype that combined aspects from ovariectomy or dihydrotestosterone treatment alone but a metabolic phenotype that resembled letrozole-treated mice

Because many different mouse models were explored, each with its own unique reproductive and metabolic phenotypes, a chart was created to compare and contrast the five different models.

For the reproductive phenotype, experimental mice of all cohorts were acyclic, but the 4mm and 10mm DHT mice had lowered ovarian weight, contrasting with the LET mice that had increased ovarian weight. DHT-only mice also had no changes in LH levels **(Table 1)**. OVX + DHT mice lacked ovaries and had severely elevated LH like the OVX mice. OVX + DHT mice also had elevated androgen levels similar to the 4mm DHT and LET mice but not as severe as the 10mm DHT mice **(Table 1)**.

For the metabolic phenotype, OVX + DHT mice gained significantly more weight compared to OVX or DHT alone. The degree of weight gain was similar to what was seen for the LET model **(Table 1)**. OVX + DHT mice also had marked abdominal adiposity and dysglycemia that resembled LET mice. Although OVX mice and 4mm DHT mice had slightly elevated levels of insulin, the degree of hyperinsulinemia was much more severe for OVX + DHT mice and resembled LET mice. Finally, insulin resistance was only seen in OVX + DHT mice and LET mice **(Table 1)**.

Table 1: Reproductive and metabolic phenotypes of ovariectomized, dihydrotestosterone, ovariectomized and dihydrotestosterone, and letrozole mouse models. Reproductive phenotypes assessed included acyclicity, change in ovary weight, elevated luteinizing hormone (LH), and hyperandrogenemia. Metabolic phenotypes assessed included weight gain, abdominal adiposity, dysglycemia (fasting blood glucose; FBG), hyperinsulinemia, and insulin resistance. ✓ = presence of phenotype, X = absence of phenotype, ↑ = slight increase in phenotype, ↑↑ = marked increase in phenotype, ↓ = slight decrease in phenotype. Two symbols separated by a semi-colon indicate differences between OVX or DHT mice in the OVX + DHT cohort (first symbol) and OVX or DHT mice in OVX-only or DHT-only cohorts (second symbol).

	OVX	4mm DHT	10mm DHT	OVX + DHT	LET
Reproductive phenotype					
Acyclicity	✓	✓	✓	✓	✓
Change in ovary weight	X	↓	↓	X	↑
Elevated LH	↑↑	X	X	↑↑	↑↑
Hyperandrogenemia	X	↑	↑↑	↑	↑
Metabolic phenotype					
Weight gain	X ; ↑	X	↑	↑↑	↑↑
Abdominal adiposity	↑	↑	↑	↑↑	↑↑
Elevated FBG	X ; ↑	↑ ; X	X	↑	↑
Elevated insulin	↑	↑	X	↑↑	↑↑
Insulin resistance	X	X	X	✓	✓

CHAPTER I: DISCUSSION

Short-term dihydrotestosterone-treatment induced reproductive dysfunction but only a mild metabolic phenotype in mice

Because the majority of published DHT mouse models utilize prolonged (3 month) DHT treatment, we focused on characterizing the effects of 5 week DHT treatment at PCOS-like (4mm) and male-like (10mm) levels in order to compare to the 5 week LET model. Previous studies tested the 4mm dose of DHT in 8-week-old adult mice and reported a 2-fold increase in serum DHT levels, impaired cyclicity, decreased glucose tolerance, hyperinsulinemia, and insulin resistance, but no dysglycemia, changes to body composition, or weight gain after 3 months of treatment[78–80]. Our results demonstrated that 5 weeks of 4mm DHT treatment in 4-week-old pubertal mice led to acyclicity, slight hyperinsulinemia, and a lack of dysglycemia or weight gain, similar to the previous studies (**Figure 3E** and **Figure 4A, C&D**). In contrast, insulin resistance was not present, and the 4mm dose of DHT led to a 7-fold rather than a 2-fold increase in serum DHT (**Figure 3C** and **Figure 4E&F**). 4mm DHT mice also had lowered ovarian weight, reflecting what is typically seen in DHT rodent models (**Figure 3D**)[76,88,89].

3-month treatment with the 10mm dose of DHT has also been previously tested in 3-week-old pubertal mice and found to induce acyclicity, a 9-fold increase in serum DHT, increased weight gain, but no changes in ovary weight or parametrial fat and a lack of dysglycemia, hyperinsulinemia, and insulin resistance[74]. Our results found that 5 weeks of 10mm DHT treatment in 4-week-old pubertal mice led to acyclicity and a lack of dysglycemia and insulin resistance, similar to the previous study (**Figure 3E** and **Figure 4C, E&F**). However, our

mice had a 13-fold increase in serum DHT and decreased ovarian weight. The mice also were not hyperinsulinemic (**Figure 3C&D** and **Figure 4D**). Because DHT was administered at such a high dose, it is possible that there was strong negative feedback onto the HPG axis to decrease estrogen production, which would lead to decreased estrogen levels in the 10mm DHT mice. However, previous studies using this dose found no significant difference in serum or intraovarian E2 levels when comparing 10mm DHT mice to placebo mice[74,76]. Additionally, uterine size is used as a proxy for estrogen action[90], and upon visual inspection, the uteri of 10mm DHT mice were not thread-like (data not shown) the way uteri of OVX (estrogen deficient) mice are. The previous 10mm DHT study also revealed no difference in uterine weight[76]. Thus, estrogen levels appear to be normal in 10mm DHT mice, and the phenotypes observed are presumably due to hyperandrogenism.

Overall, short-term treatment with either a 4mm or 10mm dose of DHT from puberty to adulthood led to reproductive dysfunction including acyclicity and decreased ovarian weight (**Figure 3**). However, only a mild metabolic phenotype was observed, suggesting that short-term hyperandrogenism alone does not induce insulin resistance. This contrasts with multiple studies that reported insulin resistance in DHT-treated rodents[78,91]. However, differences between the phenotypes of our cohorts compared to previous studies may be attributed to the duration of DHT treatment and the age of the mice at pellet implantation.

Ovariectomized and dihydrotestosterone-treated mice develop strong metabolic dysfunction that resembles letrozole-treated mice

After demonstrating that hyperandrogenism alone was not sufficient to induce insulin resistance in our DHT pilot studies, we tested if a combination of hyperandrogenism from DHT treatment and estrogen deficiency from OVX would cause insulin resistance similar to the LET model. Our results demonstrated that OVX + DHT led to a severe metabolic phenotype that was not observed in OVX or DHT alone (**Table 1**). OVX + DHT mice had significantly higher weight gain after just one week of treatment, and by week 5, they were 5-6 g heavier and gained at least 30% more weight compared to control groups (**Figure 4A&B**). Significant abdominal adiposity and dysglycemia was observed in OVX + DHT mice along with severe hyperinsulinemia and, most importantly, insulin resistance, which was not seen in DHT-only or OVX-only mice (**Figure 4C-F**). Overall, OVX + DHT mice have a combination and severity of metabolic phenotypes that strongly resemble LET mice, including weight gain, abdominal adiposity, dysglycemia, hyperinsulinemia, and insulin resistance (**Table 1**).

Insulin resistance is speculated to develop in skeletal muscle for diseases such as type 2 diabetes, obesity, and PCOS[92–94]. Results from an ex-vivo insulin challenge demonstrated that between liver, skeletal muscle, and adipose tissue, only skeletal muscle from LET mice had impaired insulin receptor signaling after 5 weeks of treatment[83]. Given the many metabolic similarities that OVX + DHT and LET mice share, it would be valuable to examine insulin signaling in skeletal muscle of OVX + DHT mice compared to DHT or OVX mice to better understand the mechanisms behind insulin resistance in PCOS and the role that hormones may play in its development.

There are only a few reported studies involving OVX + DHT treatment. Our results aligned with a previous study that reported that OVX + DHT mice had elevated FBG and glucose intolerance relative to OVX + P or SHAM + P. These mice also had increased weight gain that was similar to OVX + P[95]. Another study found that OVX + DHT mice had LH pulses that resembled LET mice, as they were elevated compared to intact placebo mice but lower than OVX-only mice[96]. We did observe a few inconsistencies in our SHAM + DHT and OVX + P control groups compared to DHT-only and OVX-only cohorts. For example, OVX + P mice did not have significant weight gain, which is characteristic of OVX mice, and SHAM + DHT mice had dysglycemia, which was not seen in the 4mm DHT pilot (**Table 1**)[84,85]. These differences may be attributed to the increased stress that the mice experienced from the double surgeries that they underwent compared to the single surgery in OVX-only or DHT-only models. Additionally, fecal samples were collected twice per week from the mice, and the additional work with the mice may have caused more stress. Nonetheless, our results provided novel information regarding the metabolic phenotypes in the OVX + DHT mouse model and highlight it as a viable model to study the role of sex hormones in metabolic dysregulation, particularly insulin resistance.

Estrogen deficiency combined with a second insult may be the key to insulin resistance in PCOS

Up until now, there has been a large focus on hyperandrogenism and its connection to PCOS metabolic phenotypes such as insulin resistance[97–100]. However, results from our DHT pilots and OVX + DHT cohort demonstrated that hyperandrogenism alone may not cause insulin resistance and that estrogen's role in insulin resistance should also be investigated. FSH levels are usually low-to-normal in women with PCOS, likely due to negative feedback on the hypothalamus and pituitary by elevated androgen levels[57,101–104]. Additionally, anovulatory women with PCOS tend to be acyclic and have follicular-phase levels of estradiol. Their ovaries also have smaller follicles, which were found to have decreased aromatase expression and estradiol production[105–107]. This provides evidence of potential estrogen deficiency in women with PCOS.

Estrogen is known to have a strong protective effect on metabolism, as OVX mice have increased weight gain and adiposity along with dysglycemia and hyperinsulinemia[84,85,108]. OVX mice are not insulin resistant, demonstrating that estrogen deficiency alone does not induce insulin resistance[84,85]. However, it has long been known that OVX mice that are fed a high fat diet (HFD) develop insulin resistance and that this can be attenuated by E2 replacement therapy[84,109,110]. Insulin resistance is also seen in the LET and OVX + DHT models, which are both estrogen deficient and hyperandrogenic[83]. In all of these models, insulin resistance results from combining estrogen deficiency with a second insult. The loss of estrogen's protective effects on metabolism may cause a state of vulnerability that alone is not enough to induce insulin resistance. However, a second insult such as HFD or hyperandrogenism may exploit the state of vulnerability left by estrogen deficiency and contribute to insulin resistance. In the

future, it would be interesting to test if E2 replacement therapy can prevent insulin resistance in LET or OVX + DHT mice. Overall, more studies should be conducted to characterize the role of estrogen in insulin resistance.

Chapter 1 is currently being prepared for submission for publication of the material. Chen, Annie; Sau, Lillian; Cui, Laura J; Rizk, Maryan; Thackray, Varykina. The thesis author was the primary investigator and author of this material.

CHAPTER II: INTRODUCTION

The Gut Microbiome and PCOS

The gastrointestinal tract is home to the gut microbiome, a complex ecosystem of bacteria, archaea, fungi, and viruses that are important for human health[111–113]. Gut microbes produce metabolites such as short chain fatty acids (SCFA) and secondary bile acids that can alter host energy harvesting and act as ligands to various host cellular receptors[114–117]. The gut microbiome also has a variety of functions outside of metabolism, such as in immune cell maturation and maintenance of the intestinal epithelial barrier[118–120]. Many environmental and host factors influence gut microbial composition, including diet, host genetics, and hormones[121,122].

Because of the codependent relationship between the gut microbiome and its host, there have been many efforts to investigate whether gut microbes are involved in disease. Studies have shown that the gut microbiota composition of individuals with metabolic syndrome differ from healthy individuals[123–126]. In two studies, germ-free mice that received fecal microbiota transplant with stool from obese human donors had metabolic dysregulation compared to those that received stool from lean human donors[127,128]. Furthermore, cohousing these two groups of mice together actually prevented metabolic dysregulation in mice that had been transplanted with stool with obese donors, demonstrating a protective effect[128]. These findings suggest that not only is the gut microbiome altered in a diseased state, but it may even play a causal role in disease.

With this, researchers began looking into whether there is a relationship between the gut microbiome and PCOS, a disease associated with a strong metabolic phenotype[58–62]. Initial clinical studies demonstrated differences in the gut microbiome between women with and without PCOS across a range of different ages and BMI[129–136]. Some studies found changes in alpha diversity (overall community species richness)[129–131,133] and beta diversity (changes in overall microbial composition between individuals)[129–132,136]. Others found specific bacteria, such as *Prevotella* genus and *Tenericutes* phylum, that were significantly changed in individuals with PCOS[129,134,136,137]. A recurring finding in multiple studies was that testosterone levels correlated with these gut microbial changes in PCOS[130–132,135]. Some studies found that hyperandrogenism correlated with changes in alpha and beta diversity[130–132], while others found a correlation between testosterone levels and changes in *Paraprevotella* abundance[133–135]. Together, these findings provide strong support that changes in the gut microbiome are associated with PCOS pathology, with testosterone as a potential driving factor.

Aside from clinical studies, many rodent studies reported similar findings in multiple different PCOS rodent models, including LET and prenatal androgen exposure models[87,138–140]. LET mice have decreased alpha diversity and changed beta diversity along with changes in the abundance of *Bacteroidetes* and *Firmicutes* bacteria[87]. Moreover, cohousing LET pubertal mice with placebo mice to expose them to healthy gut microbiota was found to protect against the development of PCOS-like reproductive and metabolic dysfunctions normally seen in LET mice[138]. These findings echo those seen in the clinical studies and further suggest that altering or improving gut microbiome dysbiosis may help alleviate PCOS symptoms.

Dietary Fiber

An important function of the gut microbiome is the production of SCFA from soluble fiber, which can be readily absorbed by the intestine. Since the human host lacks many of the enzymes necessary to degrade dietary fiber, it is fermented by gut microbes into SCFAs in the large intestine and cecum. *Bacteroidetes*, which primarily produce acetate and propionate, and *Firmicutes*, which produce butyrate, are two major phyla of bacteria present in the gut[116,141,142]. SCFAs are an important energy source for colonic epithelial cells and have been shown to influence proliferation of the intestinal epithelial cells (IEC), promoting IEC turnover and intestinal homeostasis.[121,143]. SCFAs also help maintain the integrity of the intestinal epithelial barrier by regulating tight junctions[144,145]. Additionally, SCFAs can influence immunity and metabolism by signaling through various G protein-coupled receptors[145–147]. Overall, changes in the abundance of certain bacteria can result in changes in the SCFAs produced, which can consequently alter host functions that are mediated by SCFAs.

As SCFAs are produced from fermentation of dietary fiber, multiple studies have been conducted to test if dietary fiber supplementation can be beneficial to human health. A notable meta-analysis of clinical trials found decreased risk of coronary heart disease, stroke, Type 2 diabetes and reduced body weight and total cholesterol with a daily intake of 25-29g of dietary fiber[148]. With regards to PCOS, a study in Iran gave women diagnosed with PCOS dietary interventions of 20g of either soluble or insoluble fiber each day. They found that women consuming soluble fiber, the type that can be fermented by microbes, saw significant improvement in hirsutism scores, testosterone levels, LDL, HDL, and total cholesterol among other metabolic parameters compared to women who consumed insoluble fiber[149]. Additionally, preliminary data generated in the Thackray lab found that LET mice have decreased amount of

total and individual SCFAs in their feces compared to placebo mice. Thus, we hypothesized that supplementing with dietary fiber would alleviate gut microbial dysbiosis and improve PCOS pathology, highlighting a noninvasive and potentially new treatment option for individuals with PCOS.

CHAPTER II: MATERIALS AND METHODS

PCOS Mouse Model

Three-week-old C57BL/6N female mice were purchased from Envigo and housed in a vivarium with an automatic 12 h light:12 h darkness cycle (light period: 6:00AM to 6:00PM). At four weeks of age, all mice were implanted subcutaneously with either a P pellet (3 mm diameter; Innovative Research of America; SC-111) or a slow-release LET pellet (3 mm diameter; 50 μg/day; Innovative Research of America; SX-999). Mice were given ad libitum access to water and either a control (Teklad Irradiated Control Diet (Con); Envigo; 130852) or a Benefiber-enriched diet (Teklad 5% Benefiber Diet (Fib); Envigo; 200354). Diet and pellet combinations created four experimental groups: P + Con, LET + Con, P + Fib, and LET + Fib (n=10/group). P or LET pellets were surgically replaced after 2.5 weeks of treatment to ensure efficacy. Mice were weighed and food intake was measured each week. All animal procedures in this study were approved by the University of California, San Diego Institutional Animal Care and Use Committee (Protocol S14011).

Estrous Cycle Assessment

Estrous cycles of the mice were determined during weeks 4-5 of treatment by light microscopy examination of vaginal cytology for seven days, as previously described[86]. Diestrus consisted predominantly of leukocytes; proestrus of nucleated epithelial cells; estrus of cornified epithelial cells; metestrus of a combination of cornified and nucleated epithelial cells along with leukocytes.

Insulin Tolerance Test

At 5 weeks of treatment, mice were fasted for 5 hours. A handheld glucometer (One Touch UltraMini; LifeScan, Inc.) was used to measure FBG at all timepoints. Tail blood was collected and FBG was measured at time point 0 before administration of an intraperitoneal injection of insulin (0.75 U/kg in sterile saline; Humulin R U-100). Blood glucose was measured at 15, 30, 45, 60, 90, and 120 minutes post insulin injection. Collected tail vein blood was used to measure fasting insulin levels in serum via ELISA at the UC Davis Mouse Metabolic Phenotyping Center.

Tissue Collection

5 weeks post implantation, mice were euthanized with 2.5% isoflurane. Terminal blood was collected from the inferior vena cava for various assays. Parametrial fat pads and ovaries were dissected and weighed.

Serum Assays

LH levels (range 0.02-75 ng/mL) were measured through radioimmunoassay and testosterone levels (range 10-1600 ng/dL) through ELISA at the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core Facility. Cholesterol and triglyceride levels were measured through enzymatic assay and C-reactive protein (CRP) levels through ELISA at the UC Davis Mouse Metabolic Phenotyping Center.

Statistical Analyses

The statistical package JMP 15 was used to analyze differences among groups. Data residuals were checked for normality and one-way ANOVA followed by post hoc Tukey-Kramer

honestly significant difference test was performed. If residuals were not normal, data underwent Box-Cox transformation, and non-parametric tests were performed if transformation did not result in normality. Statistical significance ($P<0.05$) was indicated by different letters or an asterisk.

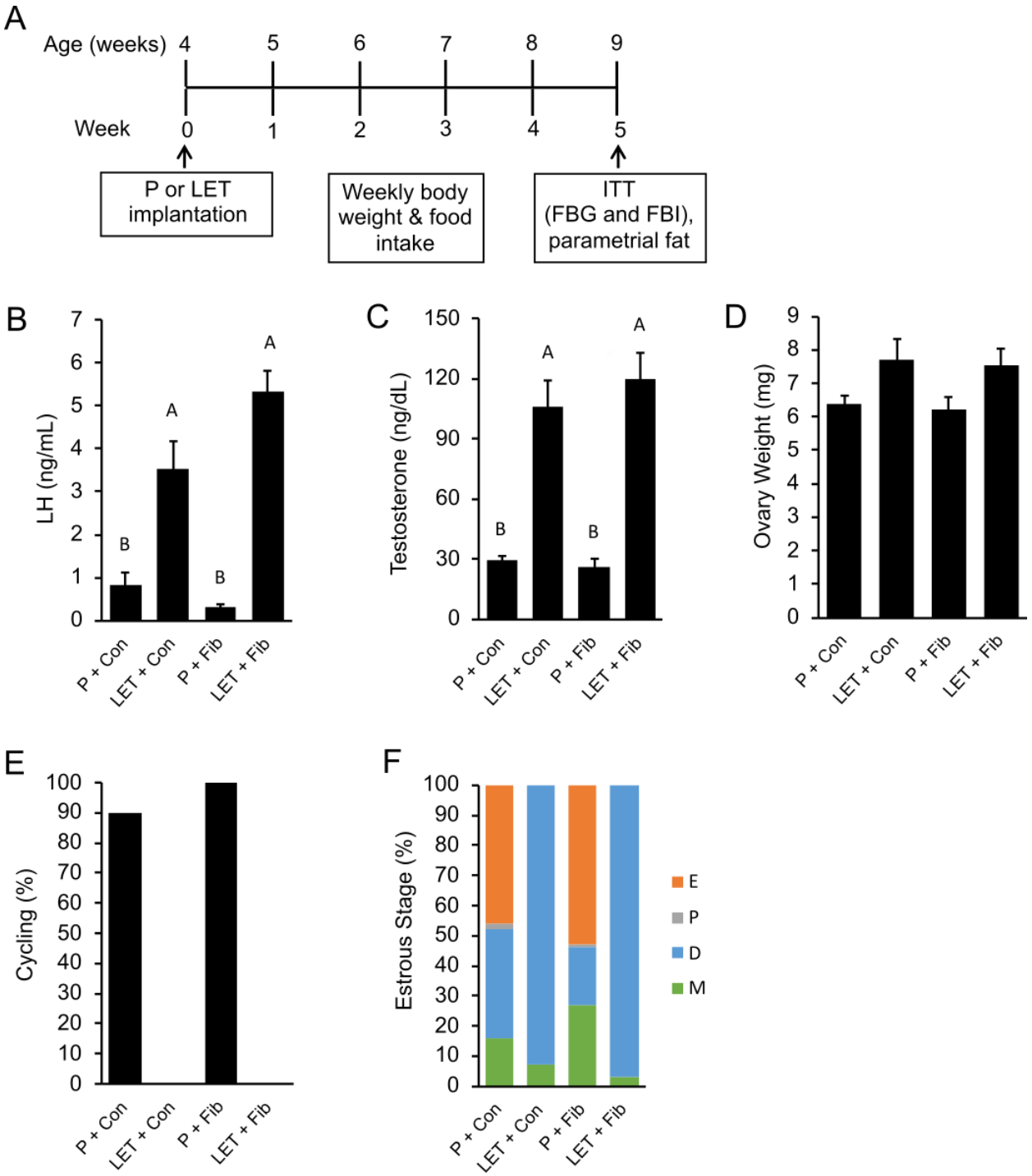
RESULTS

CHAPTER II: 5% Benefiber diet did not provide a protective effect against letrozole mice developing PCOS-like reproductive and metabolic phenotypes

Increased fiber intake did not protect against PCOS-like reproductive phenotype in letrozole-treated mice

Since increased fiber intake has been shown to protect against metabolic diseases such as diabetes and obesity[150–154], we investigated whether a diet with 5% resistant fiber (Benefiber) had a protective effect on the PCOS-like phenotype. At four weeks of age, mice were implanted with either a P or LET pellet and given either a control (Con) or 5% Benefiber (Fib) diet for five weeks (**Figure 7A**). Increased dietary fiber did not rescue elevated LH or testosterone levels in LET + Fib mice (**Figure 7B** and **Figure 7C**). Ovary weights were similar among the four groups (**Figure 7D**). In addition, LET + Fib mice did not display an improvement in estrous cyclicity and were stuck in diestrus similar to LET + Con mice (**Figure 7E** and **Figure 7F**).

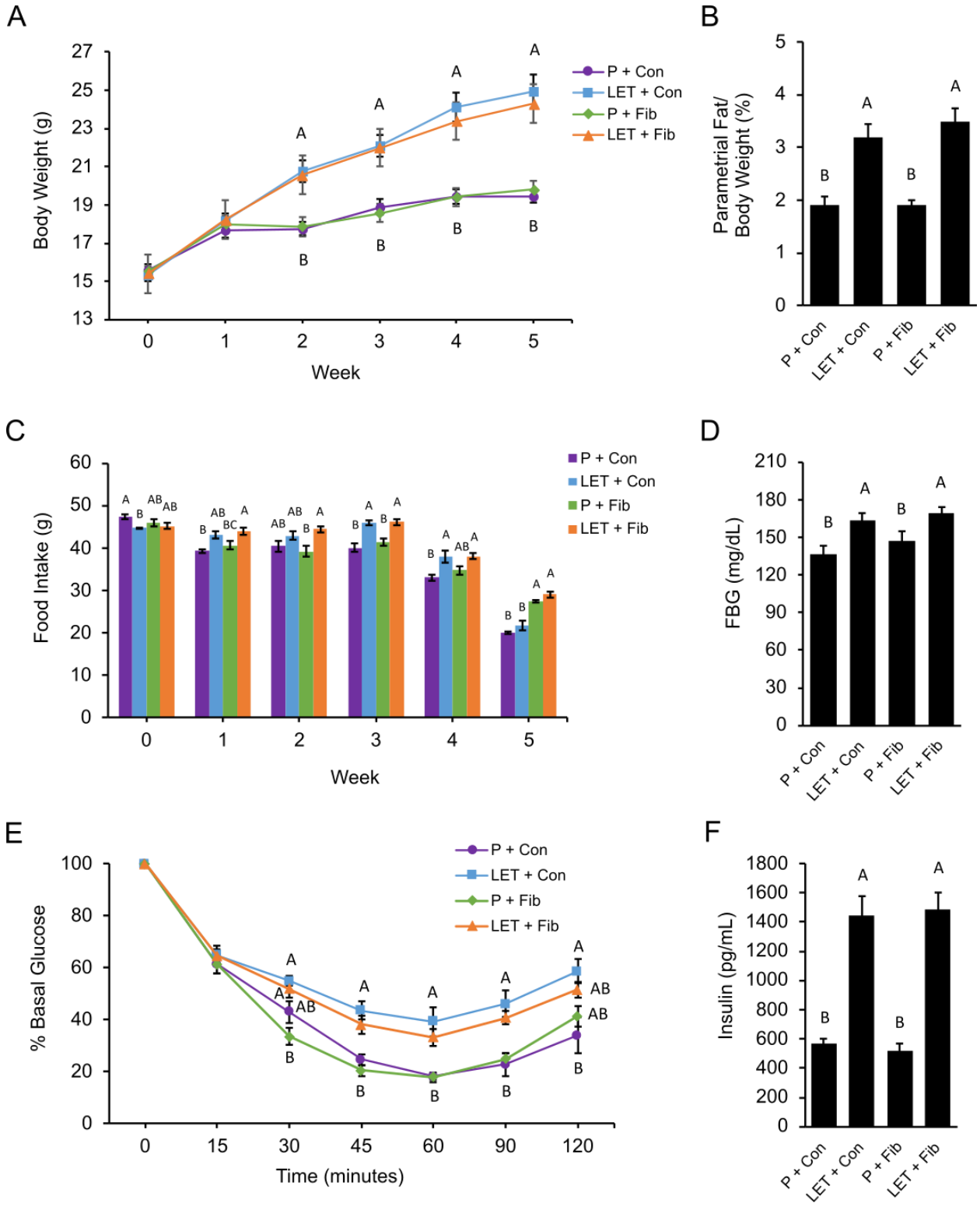
Figure 7: Consumption of a diet with 5% Benefiber did not prevent letrozole-treated (LET) mice from developing a PCOS-like reproductive phenotype. **(Panel A)** Schematic of experimental procedures: Mice were given either a placebo (P) or LET pellet implant at 4 weeks of age (n = 10 per group). Mice were fed either a control (Con) or 5% Benefiber (Fib) diet. Weekly weight assessments, an insulin tolerance test (ITT), measurement of fasting blood glucose (FBG), fasting blood insulin (FBI), and parametrial fat weight were performed. **(Panel B-C)** Treatment with 5% fiber did not protect against elevated luteinizing hormone (LH) and testosterone levels in LET + Fib mice. **(Panel D)** No significant differences were seen in ovary weight amongst the four groups. **(Panel E-F)** Treatment with 5% fiber did not restore estrous cyclicity in LET + Fib mice stuck in the diestrus stage. Standard error of the mean is represented by graph error bars. Different letters signify significant differences among groups using a one-way ANOVA followed by post-hoc comparisons with the Tukey-Kramer honestly significant difference test ($p < 0.05$).


A
Age (weeks) 4 5 6 7 8 9
Week 0 1 2 3 4 5
P or LET implantation
Weekly body weight & food intake
ITT (FBG and FBI), parametrial fat
B
LH (ng/mL)
C
Testosterone (ng/dL)
D
Ovary Weight (mg)
P + Con
LET + Con
P + Fib
LET + Fib
E
Cycling (%)
F
Estrous Stage (%)
E
P
D
M

PCOS-like metabolic phenotype in letrozole mice was not alleviated with fiber treatment

LET + Fib mice displayed no improvement in weight gain and abdominal adiposity when compared to LET + Con mice (**Figure 8A** and **Figure 8B**). No obvious changes in food intake were observed amongst the four groups, suggesting that weight gain was independent of food consumption (**Figure 8C**). Increased fiber intake also did not improve dysglycemia in LET + Fib mice (**Figure 8D**). Hyperinsulinemia and insulin intolerance were not alleviated in LET + Fib mice (**Figure 8E and Figure 8F**).

Figure 8: Treatment with 5% Benefiber did not protect letrozole (LET) mice from developing a PCOS-like metabolic phenotype. (Panel A-B) LET + Fiber (Fib) mice had similar weight gain and parametrial fat compared to LET + Control (Con) mice. **(Panel C)** There were no notable changes in food intake among the four groups. **(Panel D-F)** Increased fiber intake did not improve elevated fasting blood glucose (FBG), insulin levels or insulin intolerance in LET + Fib mice. Standard error of the mean is represented by graph error bars. Different letters signify significant differences among groups in a one-way ANOVA followed by post-hoc comparisons with the Tukey-Kramer honestly significant difference test ($p < 0.05$).


A
Body Weight (g)
Week
P + Con
LET + Con
P + Fib
LET + Fib
B
Parametrial Fat/ Body Weight (%)
C
Food Intake (g)
Week
D
FBG (mg/dL)
E
% Basal Glucose
Time (minutes)
F
Insulin (pg/mL)

Fiber treatment had a mild anti-inflammatory effect in letrozole mice

Increased fiber intake did not decrease total cholesterol or triglyceride levels in LET + Fib mice (**Figure 9A** and **Figure 9B**). However, levels of CRP, a marker of inflammation, were significantly lower in LET + Fib mice compared to LET + Con mice, highlighting a possible anti-inflammatory effect of fiber (**Figure 9C**).

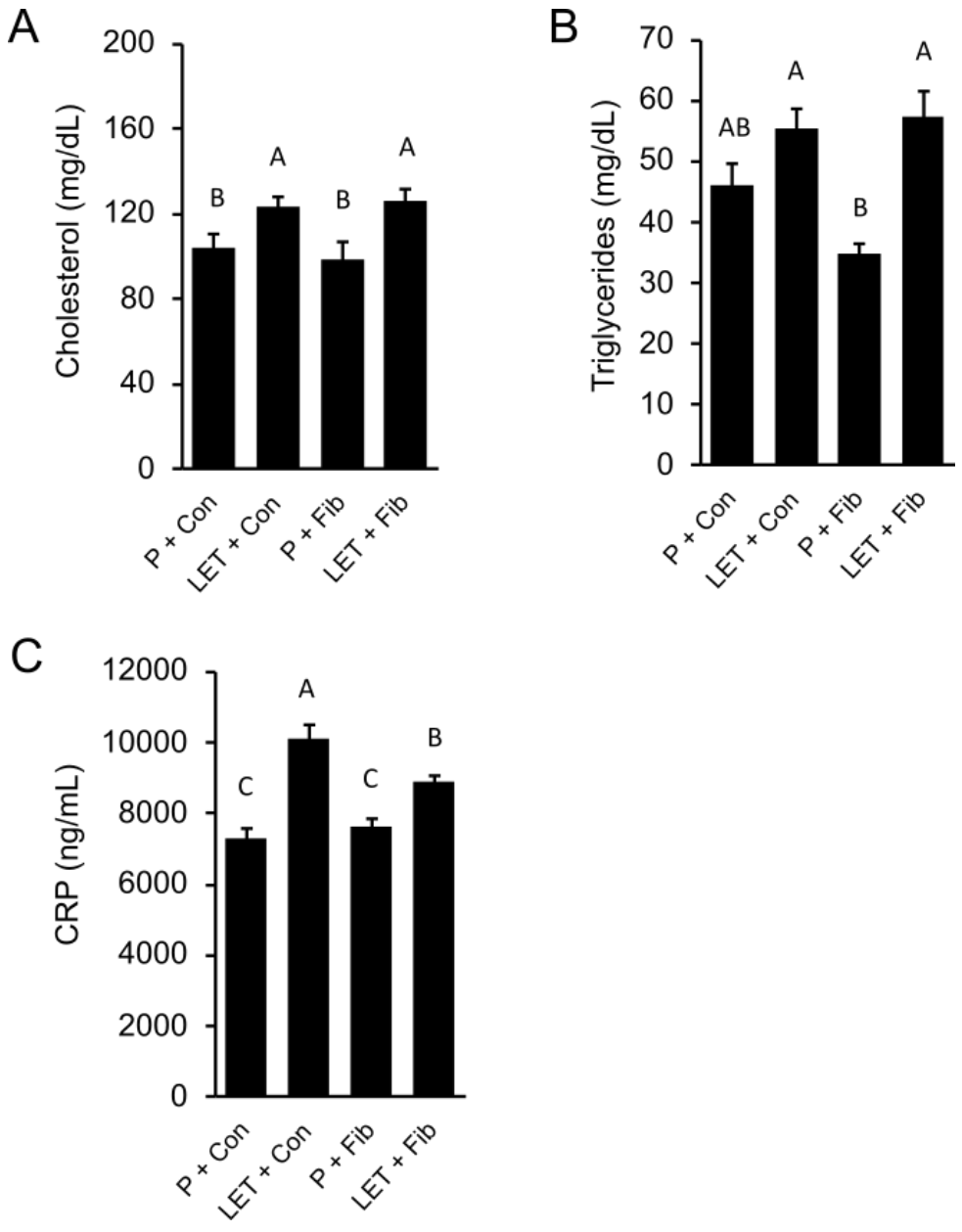


Figure 9: Treatment with 5% Benefiber slightly improved inflammation in letrozole (LET) mice. (Panel A-B) LET + Fiber (Fib) mice did not have improvements in total cholesterol or triglyceride levels. **(Panel C)** LET + Fib mice displayed decreased levels of C-Reactive Protein (CRP), a marker for inflammation, compared to LET + Control (Con) mice. Standard error of the mean is represented by graph error bars. Different letters signify significant differences among groups in a one-way ANOVA followed by post-hoc comparisons with the Tukey-Kramer honestly significant difference test ($p < 0.05$).

CHAPTER II: DISCUSSION

5% resistant fiber diet was not sufficient to prevent reproductive or metabolic phenotypes in letrozole-treated mice

Preliminary data from the Thackray lab demonstrated that LET mice had lowered total and individual SCFAs (acetate, butyrate, and proprionate) compared to placebo mice. Additionally, dietary fiber is fermented into SCFA by gut microbes and has been found to prevent and improve metabolic dysregulation[141,142,148,150,151,153–155]. Thus, we tested whether dietary supplementation with 5% resistant fiber could protect LET mice from developing PCOS reproductive and metabolic phenotypes.

For the reproductive phenotype, our results demonstrated that the fiber diet (5% Benefiber) did not normalize pathologically elevated LH and T levels or restore cyclicity in LET + Fib mice (**Figure 7B&C** and **Figure 7E**). Hemorrhagic cysts were also present on the ovaries of LET + Fib mice (data not shown). The lack of improvement in the reproductive phenotype contrasted with a previous study, which found that probiotic supplementation of inulin, another type of soluble fiber, at 0.05g/100g body weight of mouse decreased testosterone levels, increased estradiol levels, improved ovarian morphology, and lowered weight gain in DHEA-treated mice given a HFD[156]. Additionally, a clinical study demonstrated that 20g of daily resistant fiber intake improved testosterone and DHEA-S levels in Iranian women with PCOS[149].

As for the metabolic phenotype, the traits of metabolic dysfunction seen in PCOS are similar to those of metabolic disorders such as type 2 diabetes and obesity[59,62,102]. Fiber has been found to attenuate weight gain, dyslipidemia, HOMA-IR, and Hb1Ac levels, demonstrating a

protective effect against metabolic dysfunction in humans and rodents. However, our results found that LET + Fib mice had elevated weight gain and abdominal adiposity similar to LET + Con mice that was independent of food intake (**Figure 8A-C**). A diet with 5% resistant fiber also did not improve glucose homeostasis and insulin sensitivity, as LET + Fib mice had elevated FBG and FBI along with insulin resistance as demonstrated through ITT (**Figure 8D-F**). Finally, elevated cholesterol and triglyceride levels in LET + Fib mice suggested that the fiber diet did not improve dyslipidemia (**Figure 9A&B**). Overall, 5% resistant fiber diet did not attenuate the metabolic phenotype in LET mice.

Different amounts or types of fiber may exert a stronger protective effect against PCOS pathology

There are very few published PCOS mouse studies on fiber, and our study was the first to look at fiber supplementation in the LET mouse model. However, our finding that 5% resistant diet was not sufficient to protect against the development of PCOS reproductive and metabolic phenotypes in LET mice contrasted with the results from clinical and rodent studies[149,151–153,155,157,158]. One explanation for these conflicting results is that the quantity or type of fiber used for supplementation may have been insufficient or ineffective to improve gut dysbiosis. Resistant fiber in the form of over-the-counter Benefiber was chosen in this study because several previous studies found that it had positive effects on metabolic syndrome, and one study was even conducted in women with PCOS[149,158–161]. However, other types of soluble fiber such as inulin and pectin have also demonstrated positive effects on metabolic health, and many rodent studies utilize a percentage of fiber that is higher than 5%[152,157,162–166].

In the future, a higher percentage of Benefiber (e.g. 10%) should be tested along with other types of soluble fiber, and changes in gut microbial composition and SCFA abundance should also be monitored. Although further research is needed, fiber supplementation has great potential to become a noninvasive and new treatment option for women with PCOS.

Chapter 2 is currently being prepared for submission for publication of the material. Chen, Annie; Sau, Lillian; Cui, Laura J; Rizk, Maryan; Shah, Reeya S; Thackray, Varykina. The thesis author was the primary investigator and author of this material.

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