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The Effects of Testosterone on Leydig Cell Development in Male YHR+ Mice

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Abstract of the thesis of

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Leydig cells (LC) are specialized cells in the testis that develop during puberty and are responsible for producing testosterone. Luteinizing Hormone (LH) and testosterone are important for LC development. Binding of LH to its receptor (LHR) initiates the production of testosterone. Constitutively active mutations in LHR have been identified in humans resulting in puberty in males as young as 3 or 4 years of age. A transgenic mouse (YHR$^+$), was generated which mimics the constitutively active LHR by fusing the hormone, human chorionic gonadotropin, to LHR to continually activate the receptor. Testosterone levels in YHR$^+$ mice are high at neonatal ages. Previous studies in the lab have shown that YHR$^+$ mice have decreased LC numbers compared to wild type (WT) mice. It was hypothesized that high levels of testosterone at neonatal ages was responsible for the decrease in LC numbers and was causing a decrease in the proliferation of LCs. To test this hypothesis, the action of testosterone was blocked with the androgen antagonist, flutamide, and the total number of LCs as well as the number of proliferating LCs were determined. There was no significant increase in LC number or in proliferation of flutamide treated YHR$^+$ mice suggesting that other factors may be involved in the decrease in LC number. Together, these results suggest that high neonatal testosterone is not sufficient to inhibit LC development.

THE EFFECTS OF TESTOSTERONE ON LEYDIG CELL DEVELOPMENT IN MALE YHR^{+} MICE

By

STEVEN D. EBERS

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Introduction

Gonadotropins

The gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH) are secreted from the anterior lobe of the pituitary gland at puberty. After being released by the pituitary gland, the hormones have influence upon the male and female gonads (1). In males, binding of LH to its receptors, expressed in the Leydig cells within the testes is required for testosterone production. Testosterone along with FSH stimulation of the Sertoli cells is essential for sperm production and maturation (Fig. 1). Leydig cells, found in the interstitium of the testis are specialized cells that develop during puberty and start as progenitor Leydig cells (PLC) at 10 days of age in the mouse. These cells proliferate and differentiate into immature Leydig cells (ILC) at about 35 days of age, and adult Leydig cells (ALC) at about 56 days of age (Fig. 2). PLCs are spindle shaped and are found close to the seminiferous tubule. PLCs develop into ILCs, which move away from the seminiferous tubule. As it develops it acquires more smooth endoplasmic reticulum as well as cytoplasmic lipid droplets which both act to maintain the integrity of the cell and cause it to become more round shaped. ALCs, the largest of the Leydig cells are round because of their high smooth endoplasmic reticulum content although they contain no cytoplasmic lipid droplets. ALCs are responsible for the production of testosterone (2,3). Without the production of testosterone, these mice would be incompetent in producing offspring. The hormones LH, FSH, and testosterone are important for Leydig cell development.

The Yoked Hormone Receptor Transgenic Mouse (YHR^+)

In humans, a mutation can occur that causes the LH receptor to be constitutively active, even in the absence of LH. This causes testosterone to be produced even before puberty. This mutation can lead to a male reaching puberty at an age as young as 3 or 4 years. A transgenic mouse, called the YHR^+ mouse, was generated which mimics the constitutively active LH receptor by using the hormone, human chorionic gonadotropin (hCG) to continually activate the LH receptor and cause precocious puberty to occur (4) (Fig. 3). hCG is homologous to LH and can bind to the LH receptor to activate it. Previous studies in the lab have shown that these mice exhibit high levels of testosterone as early as 10 days of age but a low number of Leydig cells compared to wild type (WT) mice (Fig. 4). This reduction in the number of Leydig cells is due to a decrease in proliferation of PLCs at 10 days and 2 weeks of age. This YHR^+ mouse provides a model to study the hormonal regulation of Leydig cell development. In WT mice, a significant testosterone increase occurs at 5 weeks, whereas in the YHR^+ mouse, the increase occurs at 10 days. It is hypothesized that the high levels of testosterone at an inappropriate time causes a decrease in Leydig cell number. In this study, I have tested this hypothesis by blocking the action of testosterone with the androgen receptor antagonist, flutamide and determining its effect on Leydig cell numbers and proliferation.

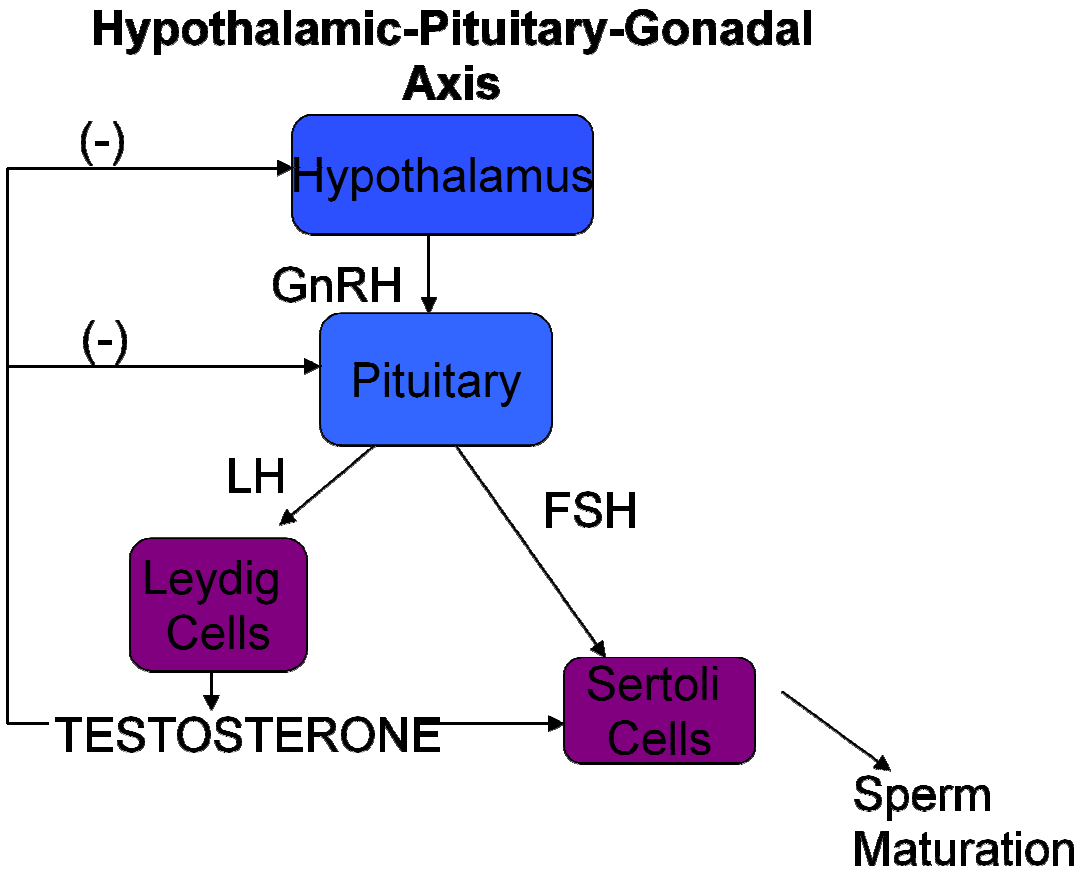


Figure 1. Gonadotropin-releasing-hormone (GnRH) is secreted from the hypothalamus and acts upon the pituitary which releases luteinizing hormone (LH) and follicle stimulating hormone (FSH) which act upon the Leydig cells and Sertoli cells, respectively. Testosterone is then released from Leydig cells and stimulates the Sertoli cells along with FSH to aid in sperm production and maturation. Testosterone exhibits negative feedback on the pituitary and the hypothalamus.

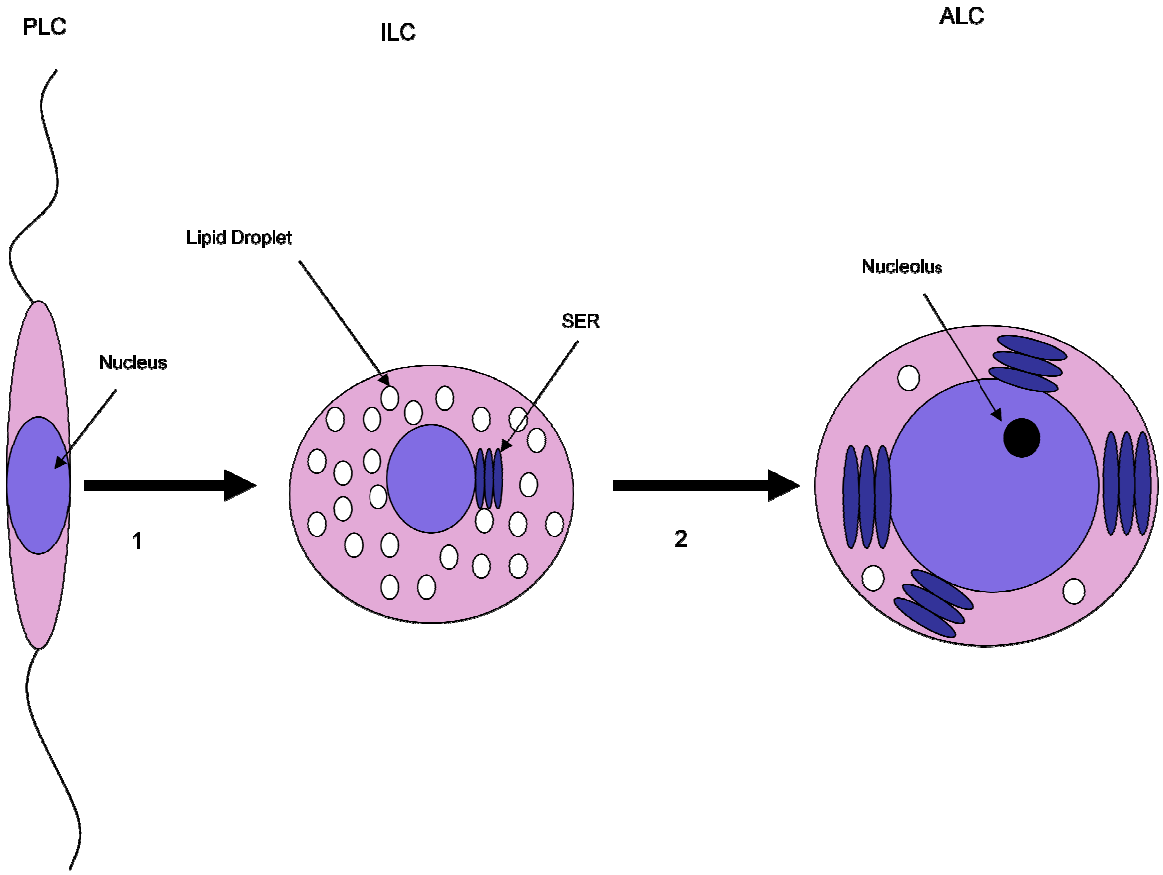


Figure 2. Leydig cell development.
Step 1 represents the progression from PLC to ILC while step 2 represents the progression of ILC into ALC. PLCs are spindle shaped and become rounded as they progress into ILCs. ILCs and ALCs are both round and cannot be distinguished morphologically. Adapted from Benton et al. (3).

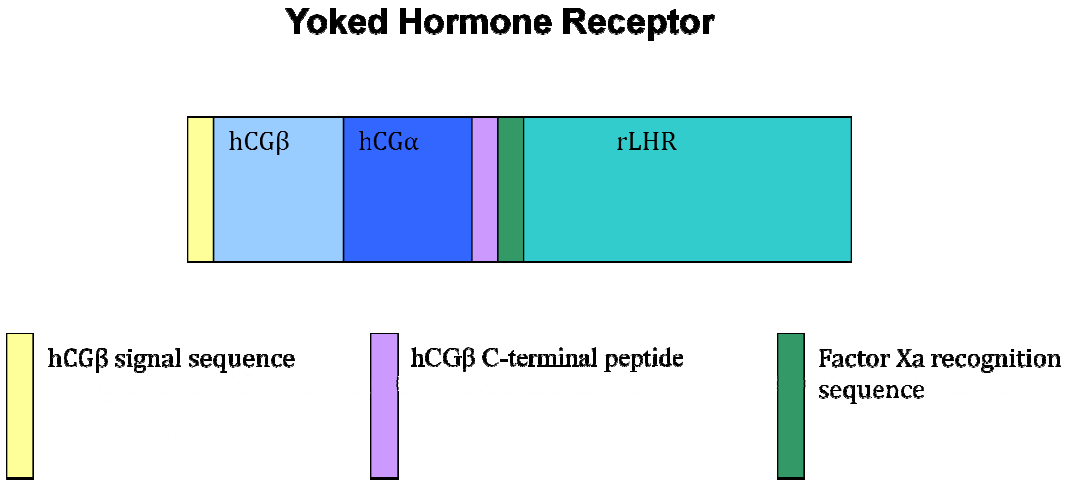


Figure 3. Schematic illustration of the yoked hormone receptor (YHR). Human chorionic gonadotropin (hCG) fused to the luteinizing hormone receptor (LHR) activates it continuously.

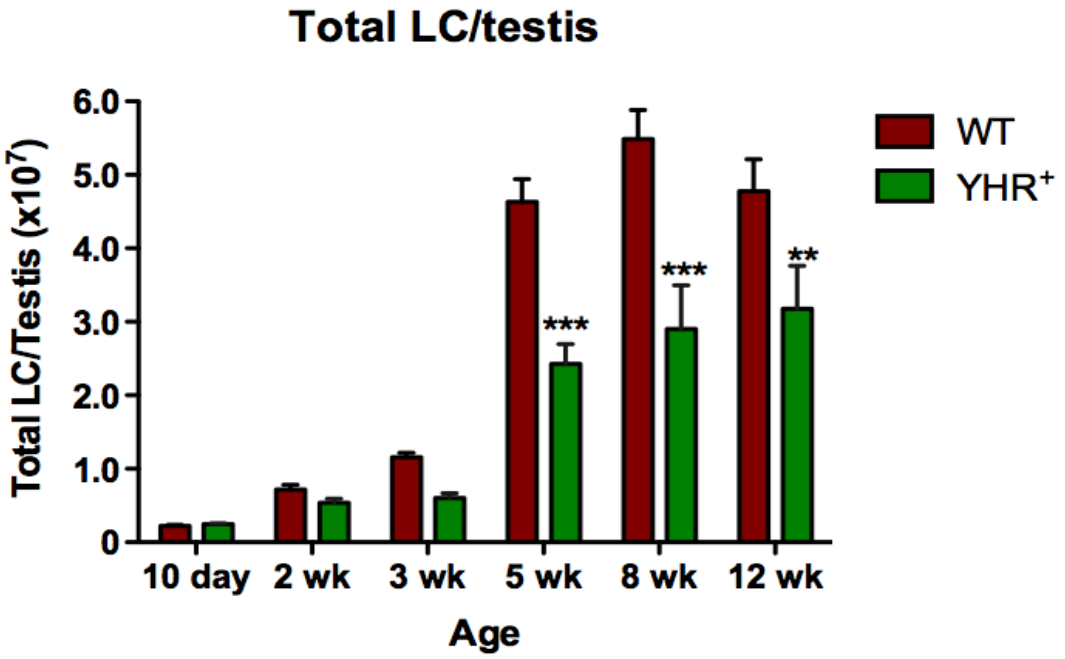


Figure 4. A decrease in Leydig cell number is seen in YHR$^+$ mice at 5, 8 and 12 weeks of age (n=5). Data from Coonce et al. (5).

Goals of the Study

1.) To block the action of testosterone with the androgen receptor antagonist, flutamide and determine its effect on Leydig cell numbers.

YHR^+ were previously found to express high levels of testosterone beginning at 10 days of age whereas WT mice exhibit a similar increase at 5 weeks of age. These YHR^+ mice also have been found to have a lower number of LC than the WT mice. In order to determine whether this high level of testosterone at neonatal ages in the YHR^+ mice is the cause of the low number of LC, testosterone action was blocked with the androgen receptor antagonist, flutamide, to examine its effects on LC numbers.

2.) To analyze the number of proliferating Leydig cells in the YHR^+ vehicle and flutamide treated animals to determine whether testosterone plays a role in proliferation.

YHR^+ mice exhibit high levels of neonatal testosterone and a low number of Leydig cells. To determine whether the high levels of testosterone at this inappropriate time is causing a decrease in LC proliferation, YHR^+ mice who had been treated with androgen antagonist were compared to control YHR^+ mice and the percent of proliferating LC were determined for each group.

Experimental Methods

Animal care and treatment

All animal studies were approved by the Institutional Animal Care and Use Committee at SIU. Mice were maintained in a conventional colony with a 12h light, 12h dark cycle and fed a standard laboratory chow and tap water ad libitum. Mice were euthanized by CO_2 asphyxiation and tissues were harvested.

Treatment of YHR^+ mice with flutamide and vehicle control

In effort to determine whether blocking the action of testosterone with flutamide will increase the number of Leydig cells, it was first necessary to determine the optimal dose of flutamide that was effective in blocking the action of testosterone. To do this, 50µg/animal·day of flutamide was injected subcutaneously above the shoulders into male YHR^+ mice on day 7, 8 and 9 to ensure the blockage of testosterone before the time period of PLC proliferation. This dose was chosen based on a previous study (6). The mice were dissected on day 10 and the effectiveness of the dose was determined by weighing the seminal vesicles. The YHR^+ mice were injected subcutaneously above the shoulders from day 7 through day 14 with 50µg/animal·day of flutamide. Another set of YHR^+ mice were treated with the vehicle control (sesame oil). The treatments occurred at these ages because Leydig cells begin to develop around day 10 and testosterone increased in YHR^+ mice at day 10.

Experimental Setup:

YHR^+ mice were treated with vehicle and flutamide starting at 7 days of age and were dissected on day 10, weeks 2, 3, 5, and 8 and their testes were collected. The testes were fixed in Bouin's solution, embedded in paraffin, and serial sections (5 µm) were obtained where sections on each slide were 25 µm apart. The sections were then stained using an antibody specific for 3β hydroxysteroid dehydrogenase 1 (3βHSD1), which is specific for Leydig cells. Blocking of endogenous peroxidase was done by using 3% hydrogen peroxide in methanol for 30 min. The sections were then incubated with normal goat blocking serum over night. The sections were then incubated with the primary antibody against 3βHSD1 at a dilution of 1:4000 for 1 h. Sections were then incubated in biotinylated secondary antibody for 10 min. Vectastain® Elite ABC reagent was then used to incubate the sections for 10 min followed by diaminobenzidine which is used to visualize the antibody-antigen complexes that had formed. Hematoxylin was then used to counterstain the sections (Fig. 5).

Quantification of Leydig cells:

5 areas in different parts of each section on each slide were randomly chosen and photographed at a magnification of 400X (Fig. 6). Only the Leydig cells that were stained with 3βHSD1 were counted within each area. Leydig cells were counted in sections that were 5µm in depth and were 25µm apart. To determine the number of Leydig cells per testis, the weight of the testis was used as an approximation of the testis volume because the density of the testis is approximately 1mg/ml (7). The weight was multiplied by the quotient of the total number of Leydig cells counted in a defined volume of $0.0047mm^3$.

This volume was calculated by multiplying the area of the box by the thickness of the section (5 µm), and multiplying this number by the total number of boxes that were counted per testis.

$$\text{LC per Testis} = \frac{\text{Total \# of LCs Counted}}{0.0047\text{mm}^3} \text{ (Weight of Testis X 10)}$$

Quantification of Proliferating Leydig cells

YHR^+ mice were injected from 7-13 days of age with either vehicle control or 50µg/animal·day of flutamide subcutaneously and with bromodeoxyuridine (BrdU), intraperitoneally, 2h prior to sacrifice at day 14. Testes were fixed in bouins solution, embedded and serial sectioned (5 µm). Immunofluorescence staining was performed with antibodies against BrdU to mark the proliferating cells and 3βHSD1 to mark Leydig cells. Endogenous peroxidase was blocked by incubating slides in 3% hydrogen peroxide for 30 min. Sections were then incubated in normal goat serum for 1 h and then were incubated over night in the primary antibody against 3βHSD1 in a 1:4000 dilution. Sections were then incubated in biotinylated secondary antibody for 1 h followed by incubation in streptavidin 594 in a 1:200 dilution for 30 min. The sections were then incubated in Vector® M.O.M.™ Mouse Ig Blocking Reagent for 3 h followed by incubation with a primary antibody against BrdU in a 1:100 dilution for 1 h. The sections were then incubated in the secondary antibody against DKαms 488 in a 1:200 dilution for 1 h. The slide was then mounted with a mounting medium with dapi. The number of BrdU and 3βHSD1 positive cells was quantified and expressed as a percent of the total number of 3βHSD1 positive LCs.

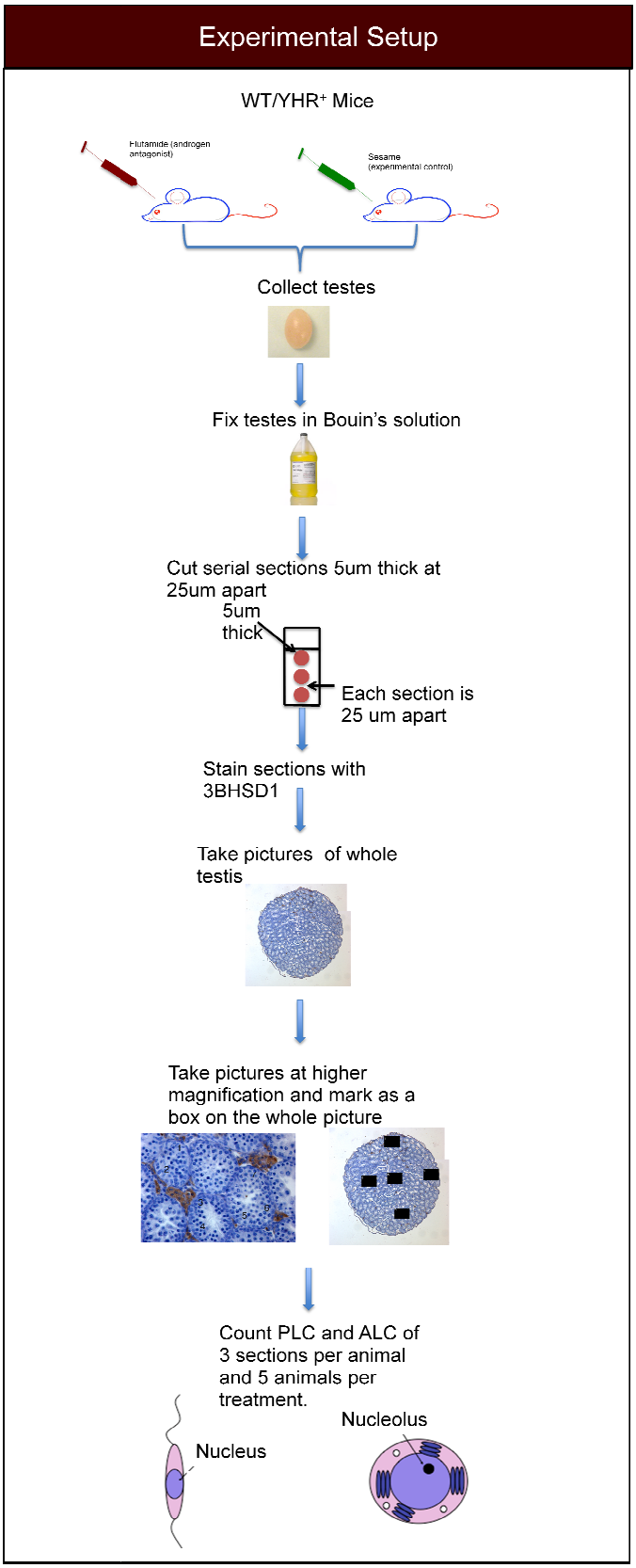
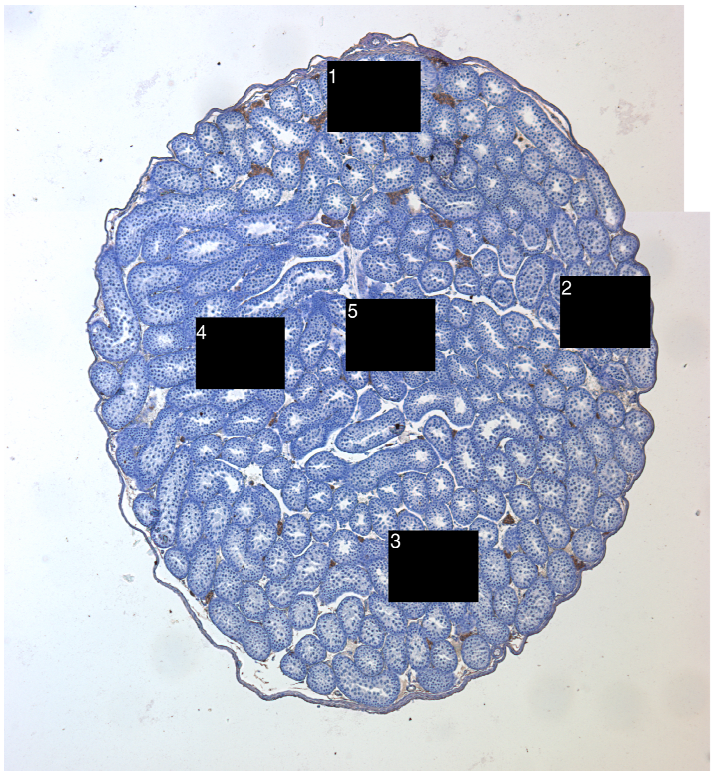


Figure 5. Experimental Setup

A.



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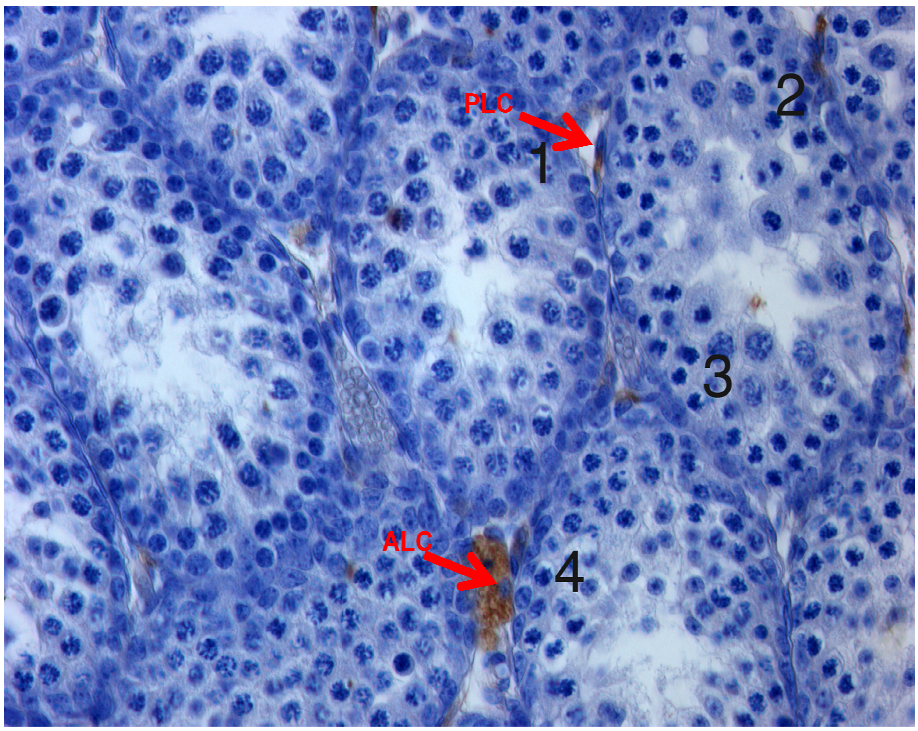


Figure 6. Sections of testis were stained with an antibody specific for 3βHSD1 to mark Leydig cells.

A. Whole testis section with boxes representing areas of counted Leydig cells.
B. Higher magnification at 400x of a representative box showing PLC and ALC.

Results and Discussion

The weight of the seminal vesicles of the YHR^+ mice, which have been treated with flutamide were compared to the weight of the seminal vesicles from WT mice and YHR^+ mice that have been treated with only vehicle. Because the seminal vesicles respond to androgens and become larger in the presence of testosterone, if the action of testosterone is blocked effectively, the weight of the YHR^+ seminal vesicles treated with flutamide should be similar to those of the WT mice. The weight of the seminal vesicles indicated that testosterone was blocked effectively at 2 and 3 weeks of age (Fig. 7). At both 2 and 3 weeks of age, the weight of the flutamide treated YHR^+ mice was significantly lower than the vehicle YHR^+ mice and was comparable to the seminal vesicle weight of the WT mice at 3 weeks. This suggests that the effect of testosterone was effectively blocked at these ages. By 5 weeks there was no significant difference in the weight of the seminal vesicles in the vehicle WT, vehicle YHR^+ and flutamide treated YHR^+ mice. This suggests that the effect of the androgen antagonist flutamide had worn off and testosterone was able to exert its normal effects by this age. This increase in testosterone effects at 5 weeks is desired because in the WT mouse a large increase of testosterone is seen at 5 weeks and is considered normal.

After quantification of LCs at 2, 3, 5 and 8 weeks of age, no significant difference in the number of LCs was seen at 2 or 3 weeks of age (Fig. 8). The number of LC was not significantly altered in the flutamide treated YHR^+ mice compared to vehicle treated YHR^+ mice. This suggests that □high levels of testosterone at neonatal ages are not sufficient to inhibit the development of LC. However the general trend is that the

number of LC in the flutamide treated YHR^+ mice is higher than that of the vehicle YHR^+ mice although not significantly higher. This suggests that testosterone may play a role in the development of LCs along with other factors.

No significant difference was found when comparing the flutamide treated YHR^+ mice to the vehicle treated YHR^+ mice at 2 weeks of age. This data suggests that the high levels of neonatal testosterone did not cause a decrease in LC proliferation (Fig. 9). Other factors may be involved in the proliferation of LC in addition to testosterone.

Further study is needed to determine which factors are contributing to LC development and proliferation in the YHR^+ mice. It has been found that FSH levels are significantly lower in YHR^+ mice compared to WT mice (4). FSH could be a contributing factor to the low numbers of LC seen in YHR^+ mice and should be studied to determine this. Another possible factor contributing to the difference in LC number could be IGF-1. IGF-1 is important for LC development and when suppressed, results in reduced numbers of LC (8). Testosterone is known to increase IGF-1 (9). Therefore, because we are blocking the action of testosterone in the flutamide treated YHR^+ mice, IGF-1 levels are likely to be decreased which could combat the expected increase in LC numbers. Because it is not known whether or not IGF-1 levels are altered in the YHR^+ mice, it would beneficial to determine IGF-1 levels the future.

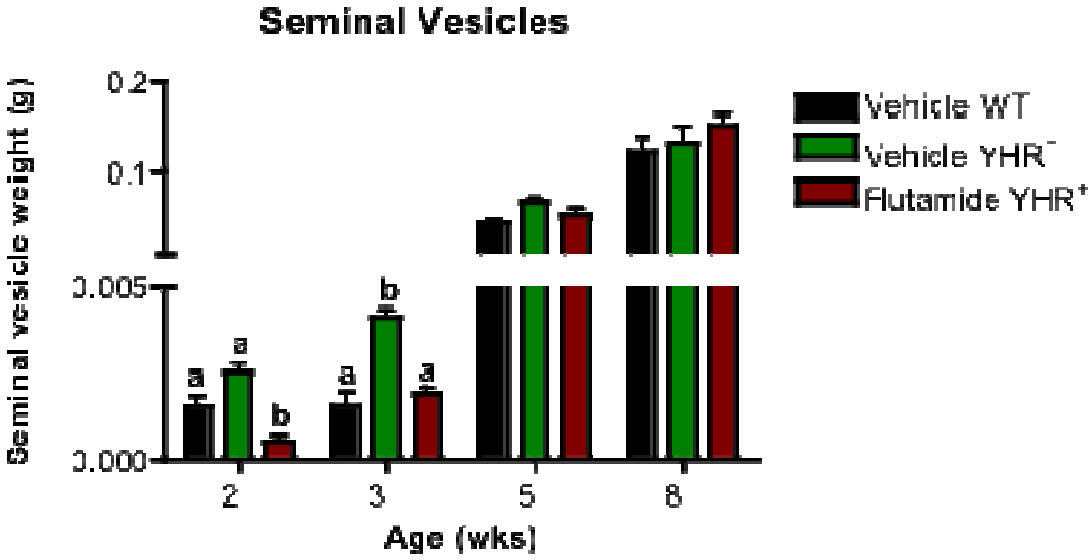
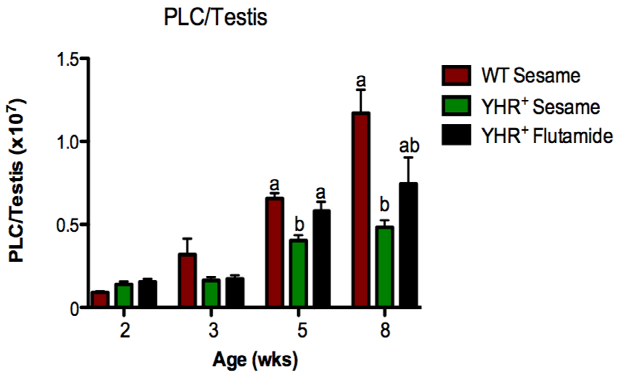


Figure 7. Data represent mean ± SEM (n=5) and were analyzed by one-way ANOVA. Different letters indicate significant differences between treatment groups ($p<0.05$).

A.



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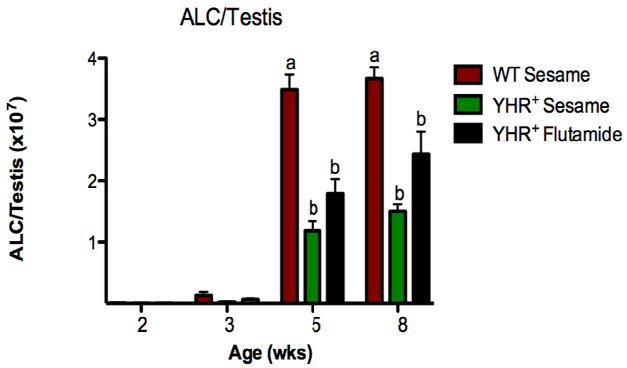

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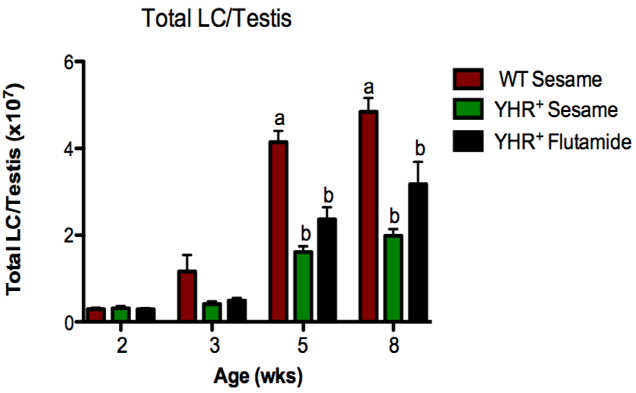

Figure 8. Data are expressed as mean ± SEM and were analyzed by one-way ANOVA (n=5). Different letter assignments indicate significant difference in treatment groups. ($p<0.05$)

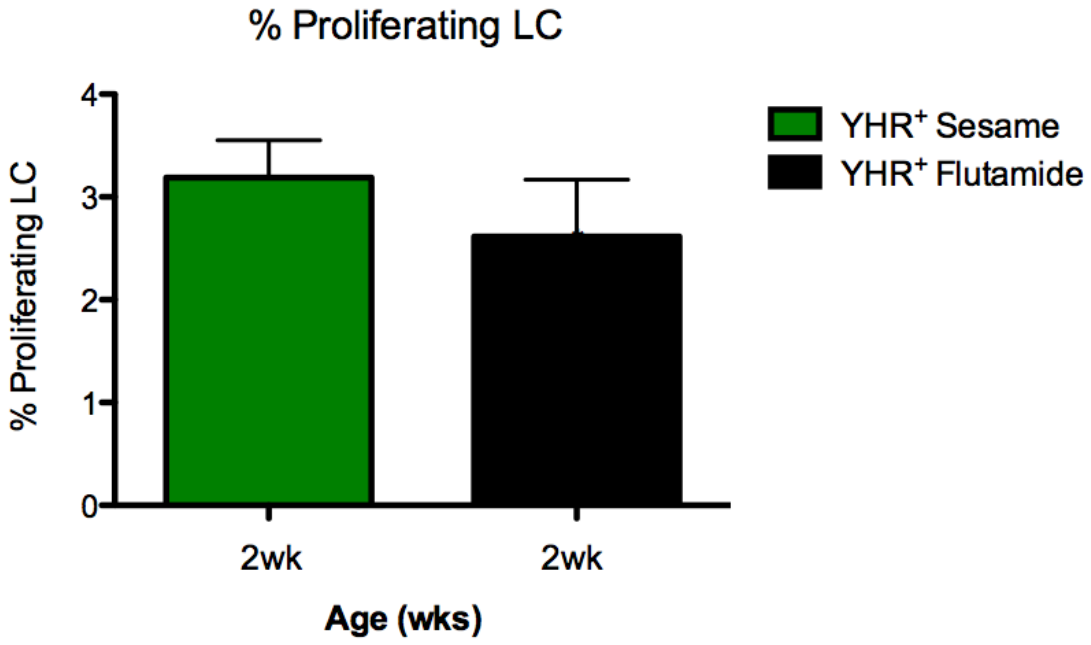

Figure 9. Data are expressed as mean ± SEM and were analyzed by t test (n=4-5). Different letter assignments indicate significant difference in treatment groups. ($p<0.05$)

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