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Effects of Growth Hormone on Testicular Testosterone Production *In Vitro* in Transgenic Mice

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EFFECTS OF GROWTH HORMONE ON TESTICULAR TESTOSTERONE PRODUCTION *IN VITRO* IN TRANSGENIC MICE

BACKGROUND:

A transgenic animal is generated by injecting foreign DNA into fertilized eggs. The eggs are then implanted in pseudopregnant females. If this DNA is incorporated into the genotype the foreign DNA may be expressed and can be inherited by offspring. When DNA for growth hormone (GH) from another species is used, the animal produces the growth hormone for the other species (1, 9).

In transgenic mice the foreign growth hormone genes cause an increased growth rate which begins usually during the fourth week following birth. The growth rate then remains increased and the adult transgenic mouse weight is 50% or more greater than their normal siblings. Transgenic mice appear to age faster than their littermates and the average life-span of transgenic mice is reduced. The male transgenic mice display a decreased fertility as compared to their siblings. This decrease in fertility is of concern in the application of transgenic technology to domestic animals such as sheep, swine, and cattle (1).

Growth hormone is secreted by the anterior pituitary and it stimulates growth of almost all cells and tissues of the body (6). It is presently thought that the actions of growth hormone are mediated by insulin-like growth factor I (IGF-I). IGF-I is an 70-amino acid straight chain peptide (4). In transgenic mice the growth hormone level may be 1000

times greater than normal, but the level of circulating IGF-I is elevated by a factor of 2 or 3 which is consistent with the hypothesis that GH effects on growth are mediated by IGF-I (7). In mice it has been shown that the synthesis of IGF-I mRNA is induced by GH at about two weeks of age, and one week later the transgenic mice begin to show an increased growth rate. IGF-I is induced before changes in weight and growth are apparent, but the initial levels of IGF-I in transgenic mice may not be elevated enough to show an increased growth response to GH (9). There are high affinity binding sites for IGF-I in the Leydig cells of the testes, and IGF-I has an effect on testosterone production in the Leydig cells. IGF-I can increase basal and human chorionic gonadotropin (hCG) stimulated testosterone production in the testes *in vitro* (8).

The Leydig cells are located in the interstices between the seminiferous tubules in the testes. These cells secrete testosterone, a steroid hormone, when stimulated by luteinizing hormone (LH) which is released from the anterior pituitary (2). Before birth and after puberty Leydig cells are numerous and produce large quantities of testosterone, but during childhood the Leydig cells are almost nonexistent (8). It has been suggested that GH, along with LH, and prolactin (PRL) is responsible for maintaining the number of LH receptors in the Leydig cells in the testes (11). In some studies it has been shown that when GH was administered to immature rats the responsiveness of the Leydig cells to

exogenous LH was increased (11).

Testosterone is essential for the growth and maintenance of the male sex organs and in mice it is also responsible for mating behavior. When male mice reach about thirty days of age their production of testosterone begins to increase sharply. This increase lasts until between 50 and 60 days of age then the production levels off and begins to slowly decline with age. It has been shown that in male mice the adult testosterone production is not constant, but is characterized by alternating periods of high and low testosterone production (3).

EXPERIMENT:

This experiment compared the testicular testosterone production response to hCG *in vitro* in transgenic mice versus their normal siblings. The amount of testosterone produced during the incubation was measured by radioimmunoassay.

All of the mice for this experiment were obtained from the Southern Illinois University at Carbondale vivarium. All of the transgenic mice in this study had a bovine growth hormone gene with a phosphoenolpyruvate carboxykinase promoter (PEPCK bGH). Phosphoenolpyruvate carboxykinase is an enzyme that is present in the body and is used during gluconeogenesis (10). When the promoter of the gene for PEPCK is combined with the bGH gene the coding for GH will be initiated in several organs including the liver and the kidneys (9).

The mice used were the results of matings between PEPCK bGH males with 1, 2, or 25 gene copies with B6C3F1 females. The mice were housed four to six per cage since weaning in a room with a controlled photoperiod of 14 hour days and 10 hour nights and a temperature of 22±1C. The mice were fed a nutritionally balanced diet and given free access to tap water. Transgenic and normal male siblings between three and five months of age were used in this experiment. For this experiment three different incubations were performed. For two of the incubations the transgenic mice were PEPCK bGH 1 copy. The mice used for the third incubation included transgenic mice which were PEPCK bGH 2 copies, and the transgenic mice used for the fourth incubation were PEPCK bGH 25 copies.

INCUBATION:

To incubate the testes the mice were weighed and then decapitated. The testes were removed and their weights before and after decapsulation were recorded (Table 1). The bodies were placed on ice. After the seminal vesicle secretions had hardened the coagulating gland was removed and the weights of the seminal vesicles were recorded (Table 1). Each testis was cut into halves and the fragments were weighed. Each fragment was placed in a 30 ml glass beaker containing 2.0 ml of buffer. The fragments were allowed to preincubate for thirty minutes in a Dubnoff shaking incubator at 32°C and 100 rpm to remove the testosterone present in the

testes. A mixture of 95% O_2 and 5% CO_2 was bubbled over the fragments as they incubated.

The four fragments from each pair of testes were then placed in 30 ml beakers containing 1.9 ml of fresh buffer and .1 ml of either 0 mIU, 2 mIU, 10 mIU, or 50 mIU/ ml of hCG. Human chorionic gonadotropin has almost the same properties as LH and stimulates the production of testosterone in the Leydig cells (6). The fragments were incubated for three hours using the same procedure as for the preincubation.

After the incubation period was over, the fragments and the media were poured into test tubes and centrifuged at 4°C at 1000 rpm for 5 minutes. The supernate was poured into plastic vials and frozen until assayed.

RADIOIMMUNOASSAY:

Radioimmunoassays were performed to measure the amount of testosterone produced by each of the fragments during the incubation. A radioimmunoassay for a hormone like testosterone involves mixing measured amounts of the hormone samples, antibody for that hormone, and aliquots of the hormone with a radiolabel. The basic principle is that the radiolabeled and unlabeled hormone will compete for binding to the antibody, and will bind with the same affinity. After an incubation period, other materials are added to separate the unbound radioactively labeled hormone from the rest of the solution. The amount of radioactive material that is left in the solution can be measured with a radiation

detector.

In tubes that contain a large amount of unlabeled hormone, little of the radioactive hormone will bind to the antibody and the reading on the radiation detector will reflect this low amount of radioactive material present. Conversely, tubes that contain little of the unlabeled hormone will have a higher amount of radioactive hormone bound to the antibody and a greater amount of radioactivity will be detected. To correlate the reading from the radiation detector with actual hormone levels, samples of a known concentration of hormone are included in the assay so that a standard curve can be generated. Other samples are also included in the assay to determine the total amount of radioactivity added to each tube (totals), the amount of the radioactively labeled hormone that was bound to other proteins present in the mixture and was not removed with the unbound labeled hormone (non-specific binding or NSB), and the total amount of binding possible (bounds). From this information the amount of hormone in each sample can be determined.

Before assaying the media obtained from the incubations, the media had to be diluted so that when 25 ul and 50 ul samples were assayed approximately 50% of the bound hormone would be radiolabeled. A binding of 50% is desirable because it is in this range that the assay tends to be the most accurate. The correct dilutions were determined by approximating the dilution required, assaying that

dilution, and then adjusting the dilution to obtain binding of approximately 50%. Before each assay the tritium labeled testosterone (H3-T) was diluted with gel-PBS so that a 100 ul aliquot in 9 ml of scintillation cocktail would have a count between 8000 and 10000 counts per minute.

To establish baseline levels for each assay, eleven tubes containing 100 ul of gel-PBS were used to measure the binding in the total, NSB, and the bound tubes. The standard curve was established by aliquoting in triplicate 100 ul samples of 5, 10, 15, 25, 50, 100, 150, 250, and 500 pg samples of the standard. Duplicate 25 ul aliquots of the Lo, Med, and Hi standard were also assayed. Twenty-five and 50 ul aliquots of each sample were measured, and then gel-PBS was added to each tube in the assay to bring the total volume of each tube to 100 ul. A 100 ul aliquot of a 1:30,000 solution of testosterone antibody was added to each tube except for the four totals tubes and the two NSB tubes to which 100 ul of gel-PBS was added. The tubes were vortexed in a multitube vortexer and 15 minutes later 100 ul of the diluted H3-T was added to all tubes. The tubes were vortexed, covered and kept for 16-20 hours at refrigerator temperature. This incubation period was necessary so that the binding between the antibody, and both the labeled and unlabeled testosterone would reach equilibrium.

After the 16-20 hour period, 1 ml of a charcoal dextran solution, was added to each of the tubes except the totals to which 1 ml of gel-PBS was added. The tubes were vortexed and

allowed to sit for 15 minutes. The fifteen minute period began when the first aliquot of charcoal was added, and the charcoal solution had to be added to all of the tubes in the first five minute period. The charcoal solution was used to bind to the H3-T that was not bound to antibody, and remove it from the solution. After the fifteen minute interval, the tubes were centrifuged for 12 minutes at 3000 rpm at 4°C.

A .6 ml aliquot of the supernate was removed from each tube and placed in a scintillation vial with 4.5 ml of scintillation cocktail. The scintillation cocktail was a toluene based solution that would emit light when it was radiated with beta emissions from the radioactive decay of the tritium. The scintillation counter would measure the amount of light emitted and display the result as counts per minute. The vials were capped, vortexed, cleaned with an antistatic solution, and placed in the scintillation counter. The vials were allowed to rest for one hour before they were counted.

The assay results were analyzed on a radioimmunoassay program developed at SIU-C for use with LOTUS 123. Any set of duplicates that had a testosterone value 2 or more times greater than the duplicate value was considered an inaccurate set and reassayed. The ng testosterone/ mg testis tissue was calculated and averaged (Table 2) and graphs of the data were made (Figs. 1-3).

RESULTS:

TABLE 1: BODY MEASUREMENTS FOR THREE STRAINS OF PEPCK bGH TRANSGENIC MICE AND NORMAL SIBLINGS

STRAIN	BODY WT.(g)	TESTES WT.(mg) DECAPSULATION BEFORE	TESTES WT.(mg) DECAPSULATION AFTER	SEMINAL VESICLE WT.(mg)	n=
1 COPY					
TRANSGENIC	54.5±5.2	256±28.7	216±30.6	295±56.5	10
NORMAL	30.8±2.5	229±20.2	203±23.6	170±28.7	8
2 COPIES					
TRANSGENIC	49.6±4.1	288±27.9	220±27.6	279±66.4	5
NORMAL	33.8±3.6	248±26.5	193±20.0	220±14.9	4
25 COPIES					
TRANSGENIC	50.8±2.2	275±20.5	231±28.2	252±40.9	5
NORMAL	27.3±2.6	231±31.4	169±18.1	196±25.3	4

TABLE 2: TESTOSTERONE PRODUCTION *IN VITRO* FOR VARIOUS STRAINS OF PEPCK bGH IN TRANSGENIC MICE AND NORMAL SIBLINGS IN RESPONSE TO hCG

STRAIN	TESTOSTERONE PRODUCTION (ng/ mg testis) 0	2	10	50 mIU hCG	n=
1 COPY					
TRANSGENIC	.33±.22	2.5±1.3	12±5.9	13±7.0	10
NORMAL	.33±.23	3.2±1.6	11±6.5	12±12	8
2 COPIES					
TRANSGENIC	.31±.29	1.3±1.6	3.8±3.3	8.4±4.5	5
NORMAL	.17±.02	.54±.23	4.0±1.2	5.9±3.7	4
25 COPIES					
TRANSGENIC	.50±.13	3.7±1.6	4.9±2.5	10±3.7	5
NORMAL	.38±.21	2.1±.92	5.4±2.6	11±3.9	4

TESTOSTERONE PRODUCTION IN INCUBATED MOUSE TESTES

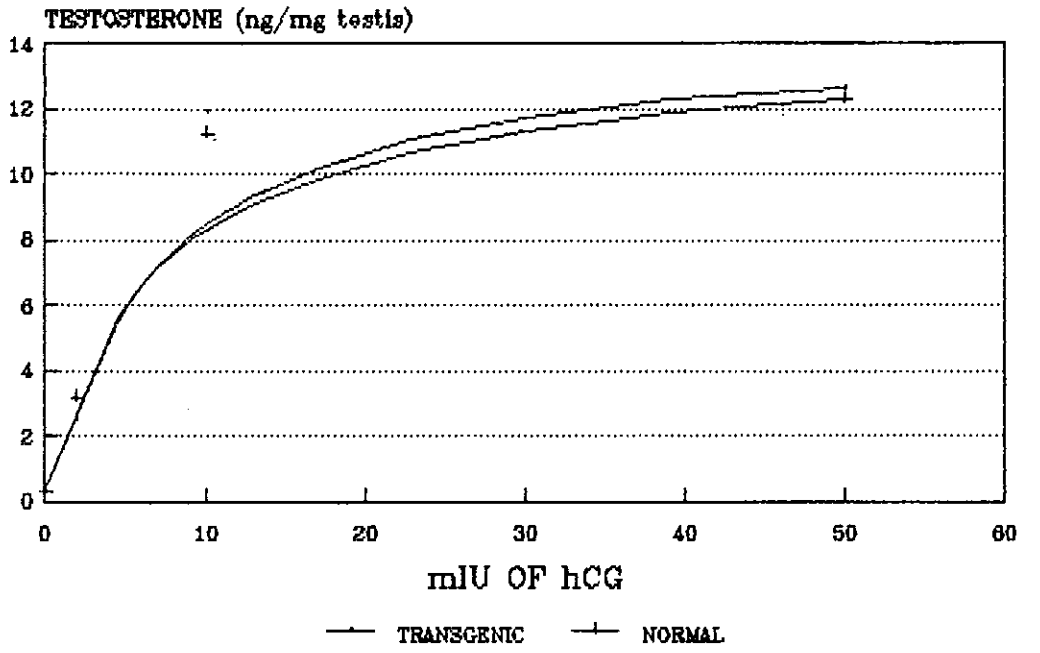

FIG. 1 PEPCK-1 COPY

TESTOSTERONE PRODUCTION IN INCUBATED MOUSE TESTES

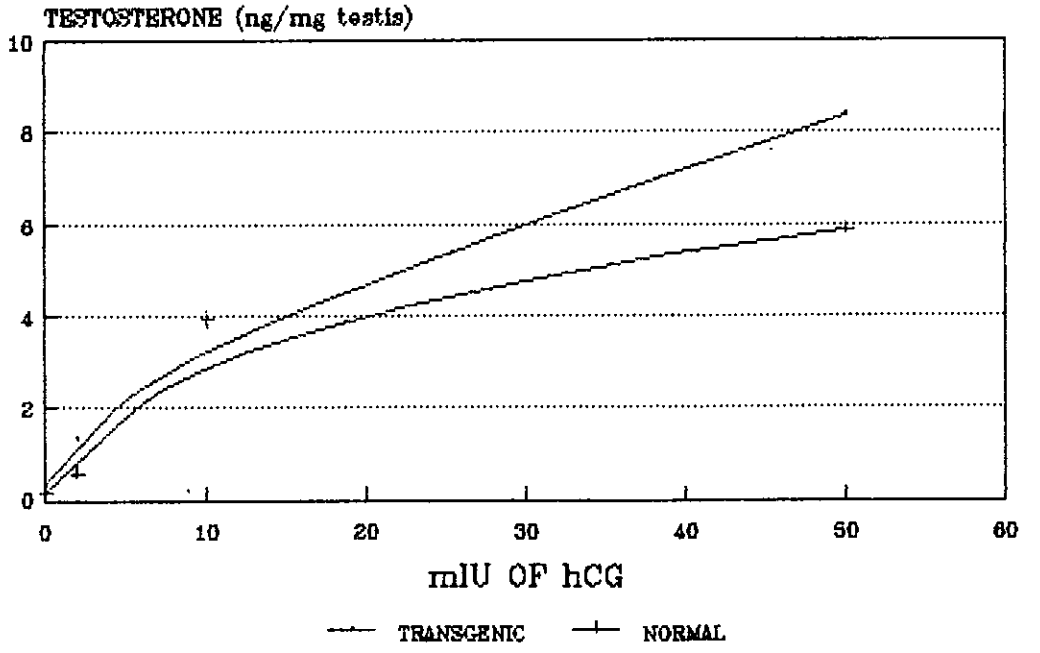

FIG. 2 PEPCK-2 COPIES

TESTOSTERONE PRODUCTION IN INCUBATED MOUSE TESTES

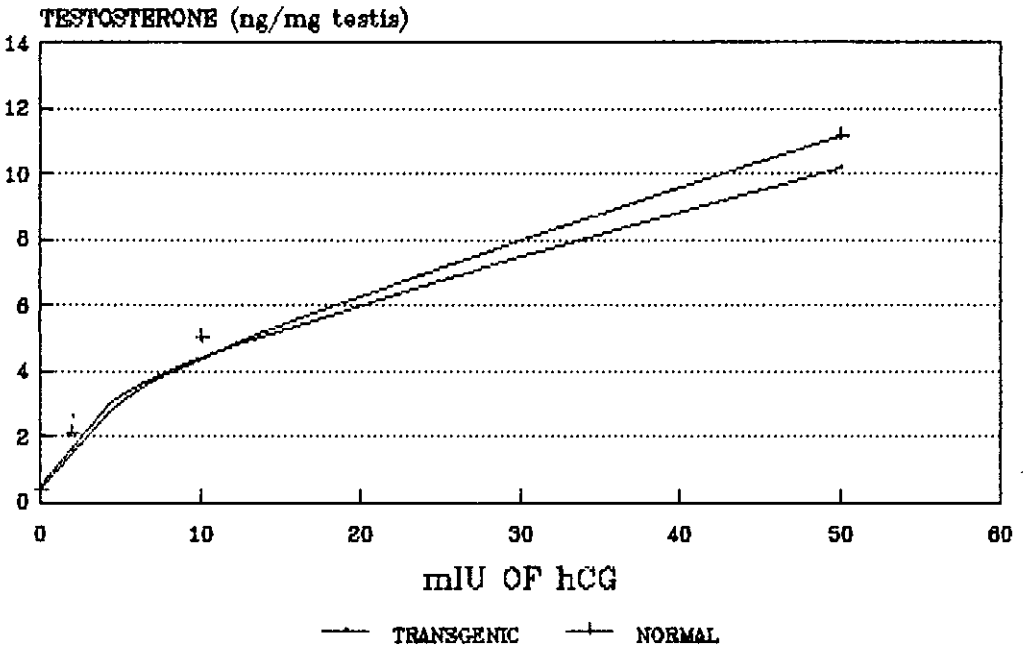

FIG. 3 PEPCK-35 COPIES

DISCUSSION:

Results of the present study indicate that the ability of the testes to produce testosterone *in vitro* and to respond to hCG stimulation was not altered in any of the examined strains of transgenic mice. The amount of testosterone produced per mg of testis tissue was not altered, but the fragments from the transgenic mice produced a larger quantity of testosterone due to the larger size of the testes in these mice. Within each strain of mice there was a large variation between the amount of testosterone produced per mg tissue derived from different animals. This could be caused by the peaks and troughs in testosterone production as discussed earlier.

As seen in table 1, the seminal vesicle weight, the testes weight, and the body weight were on the average higher in the transgenic mice for each strain than the normal siblings. The increased weight of these organs is an indication of the actions of growth hormone.

It is shown in the graphs that the strains with 2 and 25 copies of bGH did not reach peak testosterone production level at 10 mIU hCG/ ml as did the 1 copy bGH. These strains also did not reach the same maximum testosterone production as the 1 copy. This could be a result of differences in incubation conditions. The tissues for the 1 copy were incubated at separate times while the tissues from the 2 and 25 copy strains were incubated at the same time using the

same solutions. When the incubation for the 2 and 25 copy strains was performed the gas flow was accidently interrupted for no more than an hour and this could have altered the response to hCG, or altered the maximum level of testosterone production. Since all of the fragments had been exposed to the same conditions, these results were still considered valid for the purpose of comparing the differences in testosterone production in normal and transgenic siblings.

In summary, the transgenic and normal mice of the 3 strains exhibited marked differences in body weight and in testes and seminal vesicle weight. These differences in size were not reflected in the testicular testosterone production since the normal and transgenic mice both produced approximately the same amount of testosterone/ mg testis when incubated *in vitro* in the presence or in the absence of hCG.

REFERENCES

1. Bartke, A., et al. "Effects of Human Growth Hormone on Reproductive and Neuroendocrine Functions in Transgenic Mice," Unpublished work, Southern Illinois University, Carbondale, IL.

2. Bartke, A., et al., "Hormonal Interactions in Regulation of Androgen Secretion, *Biology of Reproduction* 18: 44-54 (1978).

3. Bartke, Andrzej and Susan Dalterio, "Evidence for Episodic Secretion of Testosterone in Laboratory Mice," *Steroids* 26: 749-756 (1975).

4. Berne, Robert M. and Matthew N. Levy, Physiology (St. Louis: The C.V. Mosby Company, 1988), 919.

5. Chandrashekar, V., A. Bartke, and T. Wagner, "Endogenous Human Growth Hormone (GH) Modulates the Effect of Gonadotropin-Releasing Hormone on Pituitary Function and the Gonadotropin Response to the Negative Feedback Effect of Testosterone in Adult Male Transgenic Mice Bearing Human GH Gene," *Endocrinology* 123: 2717-2722 (1988).

6. Guyton, Arthur C., Textbook of Medical Physiology (Philadelphia: W.B. Saunders Company, 1986), 877, 960, 963.

7. Lin, T., J. Blaisdell, and J.F. Haskel, "Hormonal Regulation of Type I Insulin-Like Growth Factor Receptors of Leydig Cells in Hypophysectomized Rats," *Endocrinology* 123: 134-138 (1988).

8. Lin, T., et al., "Characterization of Insulin and Insulin-Like Growth Factor I Receptors of Purified Leydig Cells and Their Role in Steroidogenesis in Primary Culture: A Comparative Study," *The Endocrine Society* 119: 1641-1647 (1986).

9. Mathews, Lawrence S., et al., "Expression of Insulin-Like Growth Factor I in Transgenic Mice with Elevated Levels of Growth Hormone is Correlated with Growth," *The Endocrine Society* 123: 433-437 (1988).

10. Stryer, Lubert, Biochemistry (New York: W.H. Freeman and Company, 1988), 439.

11. Zipf, William, Anita H. Payne, and Robert P. Kelch, "Prolactin, Growth Hormone, and Luteinizing Hormone in the Maintenance of Testicular Luteinizing Hormone Receptors," *Endocrinology* 103: 595-600 (1978).