

Comparison of Compensatory Pituitary and Testicular Responses to Hemicastration Between Prepubertal and Mature Rats

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ABSTRACT: Prepubertal (14 days old) and mature (10 to 12 months old) male rats were hemicastrated and killed at selected intervals during a 12-week period to compare the effect of age on acute and chronic pituitary and testicular responses to hemicastration. Testis weight was increased ($P < 0.05$) in hemicastrated (HC) prepubertal rats but not in mature rats compared with intact controls. After surgery, serum follicle-stimulating hormone (FSH) concentrations were elevated ($P < 0.05$) by hour 12 and day 2 in prepubertal and mature rats, respectively, and remained elevated over control levels for 4 and 8 weeks, respectively. However, the magnitude of the rise in serum FSH following hemicastration was greater in the prepubertal animals. Pituitary FSH concentrations were increased ($P < 0.05$) by day 1 in prepubertal HC rats and remained elevated through week 4. In contrast, pituitary FSH levels were unaffected ($P > 0.05$) by hemicastration in mature rats. Serum concentrations of inhibin- α were inversely related to serum FSH concentrations in prepubertal HC rats only. Serum testosterone concentrations were

reduced ($P < 0.05$) following hemicastration in both age groups but recovered to control levels within 24 hours. Neither serum nor pituitary concentrations of luteinizing hormone (LH) were altered by hemicastration ($P > 0.05$) in either age group. In addition, there were no changes ($P > 0.05$) in the concentrations of testicular LH or FSH receptors following hemicastration throughout the 12-week period. However, both receptor contents were increased ($P < 0.05$) in prepubertal HC rats in association with the increase in testis size. In summary, there were a number of age-related differences in pituitary and testicular responses to hemicastration in the rat. Also, the rapid recovery of testosterone secretion in HC animals was apparently not associated with altered LH secretion or concentrations of testicular gonadotropin receptors.

Key words: Luteinizing hormone (LH), follicle-stimulating hormone (FSH), testosterone, testis, steroidogenesis, inhibin, receptors.

J Androl 1991;12:119-125.

The control of testicular function involves complex interactions between pituitary hormones and the gonadally derived factors that control their release. At present, several testicular factors are known to be involved in feedback inhibition, including steroids and inhibin, which provides the testis with mechanisms for controlling its own activity (diZerega and Sherins, 1981). Treatments or procedures that alter gonadal homeostasis can be used to investigate testicular regulation. For example, removal of one testis (hemicastration) from prepubertal rats results in a number of changes in the remaining gonad, including testicular hypertrophy and a compensatory increase in androgen secretion (Cunningham et al, 1978; Selin and Moger, 1979). In the pubertal rat, testicular hypertrophy is not observed, but testosterone secretion returns to normal levels within a few hours after surgery (Frankel and Mock, 1982; Frankel and Wright, 1982; Frankel et al, 1984). Thus, the

hemicastrated rat has been used as a model for studying the factors that regulate testicular growth and steroidogenic activity. Although numerous studies have been conducted in several species, the exact mechanisms responsible for these compensatory changes are still not understood. The only consistently observed endocrine response to hemicastration in rats is an increase in serum follicle-stimulating hormone (FSH) concentrations (Ramirez and Sawyer, 1974; Cunningham et al, 1978; Frankel and Wright, 1982). It has been suggested that increased FSH secretion following hemicastration may mediate testicular hypertrophy responses (Cunningham et al, 1978). The factors responsible for compensatory androgen secretion have not been determined. There are no apparent changes in luteinizing hormone (LH) pulsatility or gonadotropin-releasing hormone (GnRH)-stimulated LH release in the hemicastrated male rat (Frankel and Mock, 1982), suggesting that this response may be regulated locally. In this study, we reevaluated the compensatory pituitary and testicular responses to hemicastration to answer the following questions: 1) How do concentrations of serum inhibin change in relation to altered FSH secretion?; 2) How are pituitary concentrations of FSH affected by hemicastration?; 3) Is testicular sensitivity to

This work was supported, in part, by USUHS Grant No. CO8527.

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Received for publication August 16, 1990; accepted for publication October 30, 1990.

circulating gonadotropins altered through increased LH or FSH receptor binding?; and 4) how do these pituitary and testicular responses to hemicastration differ between prepubertal and mature rats?

Materials and Methods

Animals and Treatment

Adult male (10 to 12 months old) and pregnant female Sprague-Dawley rats were purchased from Charles River Breeding Laboratories (Wilmington, MA). Females were allowed to deliver normally, and at 7 days of age, male and female pups were separated and litters normalized to 12 males per lactating female. Pups were hemicastrated (HC) at 14 days and then weaned at 21 days of age. Mature males were hemicastrated after a 10-day acclimation period.

All males were hemicastrated (HC) under methoxyflurane anesthesia (Metophane, Pitman-Moore, Inc., Washington Crossing, NJ). Hemicastration was performed through a midscrotal incision by exposing the right testis, ligating all testicular and epididymal vessels, and removing the testis and associated epididymis. The incision was closed with nonabsorbable sutures. Control intact rats were anesthetized only. Rats ($n = 7$ to 8/group) were exsanguinated under methoxyflurane anesthesia at the following times after surgery: 6, 12, 18, 24, and 48 hours; 4, 7, and 14 days; and 4, 8, and 12 weeks. To minimize stress, all rats were kept outside the procedure room until the time each animal was killed. Blood was centrifuged (2,000g for 20 minutes at 4°C) within 2 hours, and the resulting sera was collected and stored at -20°C for hormone analyses. Pituitaries and testes were collected, frozen immediately on dry ice, and stored at -80°C.

Hormone and Receptor Assays

Pituitaries were homogenized in phosphate-buffered saline (PBS)-gel buffer (0.01 mmol/L sodium phosphate, 0.14 mmol/L NaCl, 0.1% gelatin, pH 7.2) to a concentration of 5 mg/ml and centrifuged at 3,000g for 30 minutes. Supernatant was diluted with PBS-gel buffer for analysis of LH and FSH. Testes were homogenized in Tris-MgCl₂ buffer (10 mmol/L Tris-HCl, 3.8 mmol/L sodium azide, 5 mmol/L magnesium chloride, 0.1% bovine serum albumin, 10% glycerol, pH 7.4 at 20°C) to a concentration of 50 mg/ml for LH and FSH receptor analysis.

Serum and pituitary LH and FSH were quantified using radioimmunoassay (RIA) kits for rat serum provided by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK); both standard preparations were RP-2. Inter- and intra-assay coefficients of variation were 7.1% and 9.5%, and 6.7% and 9.1% for LH and FSH, respectively. Serum testosterone was analyzed using a human ¹²⁵I RIA kit (Radio Systems Laboratories, Carson, CA) previously validated for use with rat serum (Brown and Chakraborty, 1988). All samples were measured in a single assay with an intra-assay coefficient of variation of 5.9%. Testicular LH and FSH receptor concentrations were determined in crude homogenates using a previously described standard curve technique (Brown and Chakraborty, 1988). The intra- and inter-

assay coefficients of variation were 6.1% and 8.7%, and 5.9% and 7.8% for LH and FSH, respectively.

An inhibin RIA was developed against a synthetic porcine $\alpha(1-30)$ inhibin fragment (inhibin- α , N. Ling, Salk Institute, San Diego, CA). Inhibin was conjugated to keyhole limpet hemocyanin (KLH) using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC). Five milligrams of inhibin- α , 5 mg KLH, and 5 mg EDAC were dissolved in PBS (1 mol/L, pH 5.0) and reacted overnight at 4°C. The peptide conjugate was dialyzed against PBS (2 × 2 liter for 4 hours at 4°C) to remove excess coupling agent. After emulsification in Freund's complete adjuvant (Gibco Laboratories, Grand Island, NY), approximately 250 μ g peptide conjugate was injected (subscapular and intradermal) at several sites into two rabbits. Booster injections in Freund's incomplete adjuvant were given every 2 weeks for 6 weeks and then at 9, 13, and 16 weeks. Both rabbits immunized against the conjugated peptide produced antisera with final assay titers of 1:100,000 (JLB#491) and 1:200,000 (JLB#492). These antisera had similar sensitivity and specificity characteristics.

The same synthetic $\alpha(1-30)$ fragment used for antibody preparation was radioiodinated using chloramine-T. In brief, 2.5 μ g peptide was dissolved in 25 μ l 0.05 mol/L phosphate buffer (pH 7.6) and reacted with 1 mCi ¹²⁵I (Amersham Corp., Arlington Heights, IL) and 2.5 μ g chloramine-T for 60 seconds. The reaction was stopped by the addition of 10 μ g sodium metabisulfite. The reaction mixture was chromatographed on anion exchange resin (AG 2-X8, 100 to 200 mesh, BioRad Laboratories, Richmond, CA) eluted with phosphate buffer. Radioiodinated inhibin- α was diluted in assay buffer (PBS containing 0.1% bovine serum albumin, pH 7.4) to approximately 20,000 cpm/100 μ l. The assay was incubated at room temperature for 4 days with serum (10–25 μ l) and antiserum (JLB#492) added on day 1, labeled hormone added on day 2, and second antibody (sheep anti-rabbit gamma globulin) added on day 3. Separation of free from antibody-bound hormone was achieved on day 4 after addition of 1 ml PBS (0.01 mol/L, pH 7.2) and centrifugation at 3,500g for 30 minutes. Inhibin- α antiserum bound 25% to 40% of the labeled peptide with 2% nonspecific binding. Sensitivity, calculated as 95% of maximum binding, was 5 pg/tube. All samples were measured in a single assay with an intra-assay coefficient of variation of 6.7%.

Statistics

All values are expressed as the mean \pm standard error of the mean (SEM). Significant differences were determined by analysis of variance. Mean differences between each time period were determined using Student's *t* tests (Steele and Torrie, 1980).

Results

Inhibin RIA Validation

Addition of the inhibin- α peptide to pools of castrated male rat serum resulted in a mass recovery of $98.3 \pm 3.7\%$ after subtraction of endogenous activity. Displacement of ¹²⁵I-inhibin- α to JLB#492 antiserum by serial dilutions of intact male rat serum, porcine follicular fluid (pFF), and rat

testis supernatant was parallel to the inhibin- α standard curve (Fig 1). Displacement by serial dilutions of other sources of synthetic inhibin- α fragments, including porcine inhibin fragment $\alpha(1-32)$ (Peninsula Laboratories, Belmont, CA), Tyr²⁷ rat inhibin $\alpha(1-27)$, and ovine inhibin $\alpha(1-25)$ Gly-Tyr (Salk Institute), was also parallel to the inhibin- α standard curve (Fig 1). Other proteins, including rLH, rFSH and prolactin (rPrI) (NIDDK), recombinant human insulin-like growth factor (IGF-I; Amgen Biologicals, Thousand Oaks, CA), epidermal growth factor (mE F; Sigma Chemical Co., St. Louis, MO), and atrial natriuretic factor (rANF), transforming growth factor (rTGF- β), and human seminal plasma inhibin-like peptide (Peninsula Laboratories, Belmont, CA) in concentrations of 20 to 500 ng/tube did not displace antibody-bound tracer in this assay. Although binding inhibition was observed with castrate rat serum, the activity was minimal compared to that observed for intact rat serum (Fig 1).

Prepubertal Rats

The testis weight of hemicastrated rats was increased ($P < 0.05$) by day 7 compared with intact controls (Fig 2A). Although serum testosterone concentrations were initially reduced ($P < 0.05$), levels returned to those observed in intact animals 24 hours after surgery (Fig 2B). Neither serum LH (1.25 ± 0.15 ng/ml and 1.30 ± 0.21 ng/ml) nor pituitary LH (0.82 ± 0.05 μ g/mg and 0.84 ± 0.06 μ g/mg) concentrations differed ($P > 0.05$) between hemicastrated and intact rats, respectively. Similarly, testicular concentrations of LH and FSH receptors were not different ($P > 0.05$) between intact and hemicastrated rats at any time point (Figs 3A and C). However, LH and FSH receptor contents were increased ($P < 0.05$) because of the increase in testis weight (Figs 3B and D).

Temporal patterns of serum and pituitary FSH in both intact and hemicastrated rats varied throughout develop-

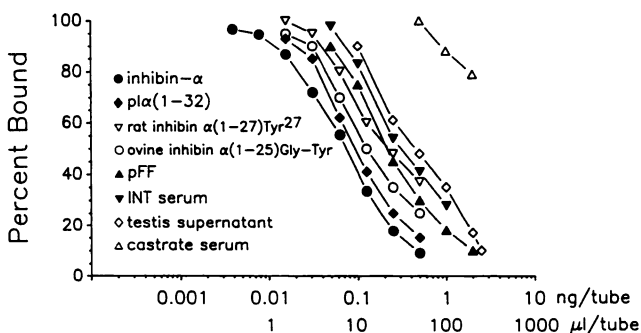


FIG. 1. Displacement of ¹²⁵I-labeled synthetic porcine inhibin (pl) fragment pl $\alpha(1-32)$ (inhibin- α) binding to inhibin antiserum by the reference preparation pl $\alpha(1-32)$, another inhibin fragment pl $\alpha(1-32)$, rat inhibin $\alpha(1-27)$ Tyr²⁷, ovine inhibin α (Gly-Tyr), porcine follicular fluid [pFF] diluted 1:20 in PBS, intact male rat serum (INT), rat testis supernatant (in PBS), and castrated male rat serum.

ment (Figs 4A and B). However, serum concentrations in hemicastrated rats increased ($P < 0.05$) above intact levels by hour 12 (78% increase), peaked on day 7 (140% increase), and then remained elevated through week 4 (Fig 4A). Similarly, pituitary FSH concentrations in hemicastrated rats also were greater ($P < 0.05$) than in intact rats between day 2 and week 4 (Fig 4B). Serum inhibin- α concentrations in hemicastrated rats were reduced ($P < 0.05$) 12 hours after surgery and remained lower than intact animals through day 14 (Fig 4C). Levels of inhibin- α also decreased with increasing age. Overall mean inhibin- α concentrations in intact rats (1.38 ± 0.04 ng/ml) were greater ($P < 0.05$) through week 4 (prepubertal) than those concentrations observed after puberty (weeks 8 and 12; 0.76 ± 0.02 ng/ml).

Mature Rats

The testis weight of mature rats was not altered ($P > 0.05$) by hemicastration. Overall mean testis weights were 1.65 ± 0.1 g and 1.73 ± 0.15 g for hemicastrated and intact rats, respectively. Serum testosterone concentrations in hemicastrated rats were reduced ($P < 0.05$) between hours 6 and 18 but returned to intact levels ($P > 0.05$) 24 hours after surgery (Fig 5A). Neither serum LH (overall mean \pm SEM: 0.75 ± 0.04 ng/ml and 0.72 ± 0.10 ng/ml) nor pituitary LH (0.73 ± 0.01 μ g/mg and 0.72 ± 0.01 μ g/mg) concentrations varied between hemicastrated and intact rats, respectively. Similarly, overall mean concentrations of testicular LH (4.24 ± 0.20 pmol/g tissue and 4.47 ± 0.06 pmol/g tissue) and FSH (6.27 ± 0.24 pmol/g and 6.68 ± 0.29 pmol/g) receptors did not differ between hemicastrated and intact rats, respectively.

Serum FSH concentrations were increased ($P < 0.05$) in hemicastrated rats between day 2 and week 8 (Fig 5B); however, pituitary FSH concentrations were unchanged (Fig 5C). The magnitude of the rise in serum FSH following hemicastration was greater in prepubertal (approximately 140%) than mature (approximately 40%) rats. Although between animal variation was considerable, there were no apparent differences ($P > 0.05$) in concentrations of inhibin- α between hemicastrated (2.59 ± 0.24 ng/ml) and intact (2.17 ± 0.20 ng/ml) rats.

Discussion

By comparing the hemicastration responses of prepubertal (14-day-old) rats with more mature (10- to 12-month-old) animals, this study differs somewhat from previous work that typically used adult rats aged 6 months or younger. These data therefore provide additional information regarding maturational changes in the pituitary-testicular response to hemicastration in this species. An increase in the size of the remaining testis was observed in prepubertal rats, which agrees with earlier studies (Cunningham et al, 1978; Selin

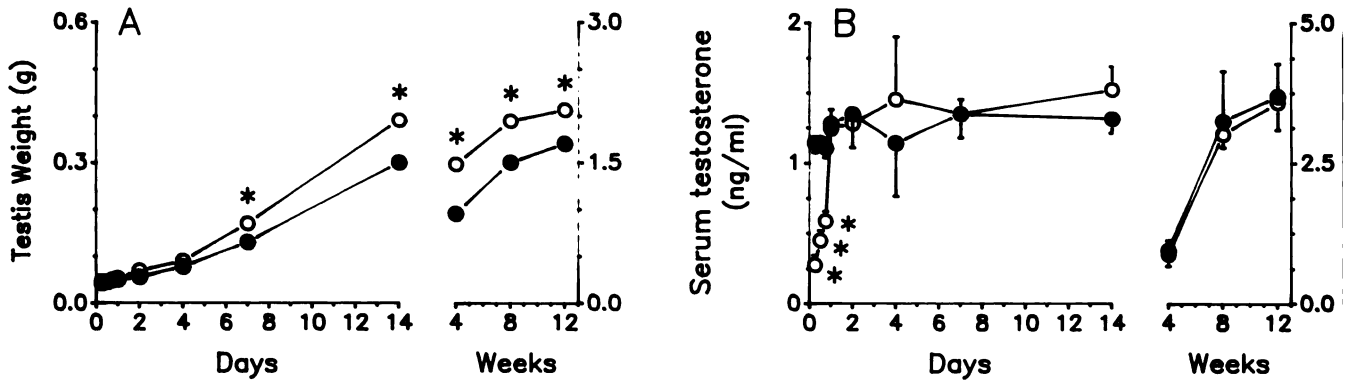


FIG. 2. Effects of hemicastration on testis weight (A) and concentrations of serum testosterone (B) in hemicastrated (open circles) and intact (filled circles) prepubertal (14 days old) male rats. Values are presented as the mean \pm SEM. For points with no SEM bars, the values fell within the symbols. Note the scale differences between left and right Y-axes. *Differs from intact control ($P < 0.05$).

and Moger, 1979). The lack of testicular hypertrophy in the mature hemicastrated group is similar to that described for younger adult rats (Cunningham et al, 1978; Frankel and Wright, 1982) but differs from the increase in testis weight observed following hemicastration in a study that used much older (about 2 years) animals (Johnson and Neaves, 1981). It is unclear why testicular hypertrophy occurs in

very young and old rats but not in animals of intermediate ages. Because of its importance in regulating Sertoli cell proliferation and growth, FSH has been implicated as the causative factor directly responsible for testicular hypertrophy in prepubertal rats (Cunningham et al, 1978; Orth, 1984). Elevated serum FSH concentrations following hemicastration have been reported previously for both prepu-

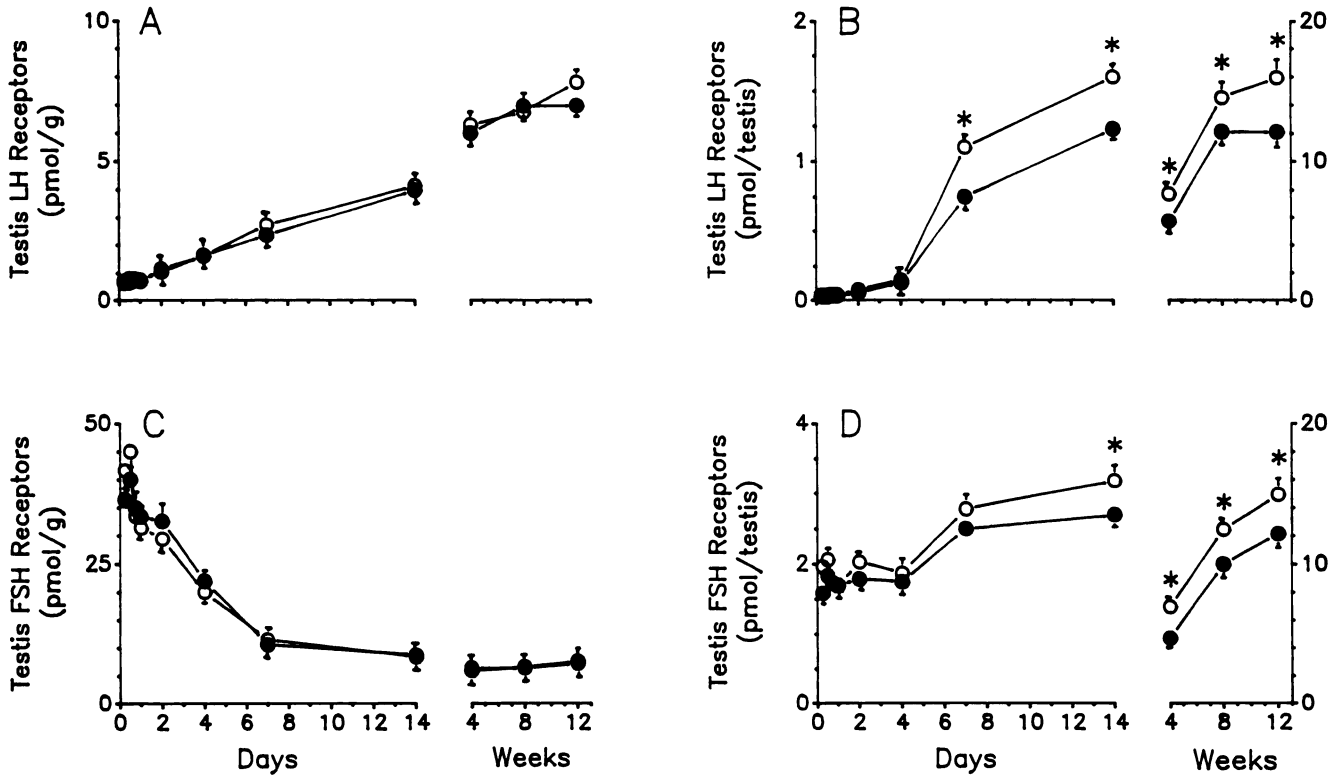


FIG. 3. Effects of hemicastration on the concentrations and contents of testicular LH (A, B) and FSH (C, D) receptors in hemicastrated (open circles) and intact (filled circles) prepubertal (14 days old) male rats. Values are presented as the mean \pm SEM. For points with no SEM bars, the values fell within the symbols. Note the scale differences in the Y-axes of Panels B and D. *Differs from intact control ($P < 0.05$).

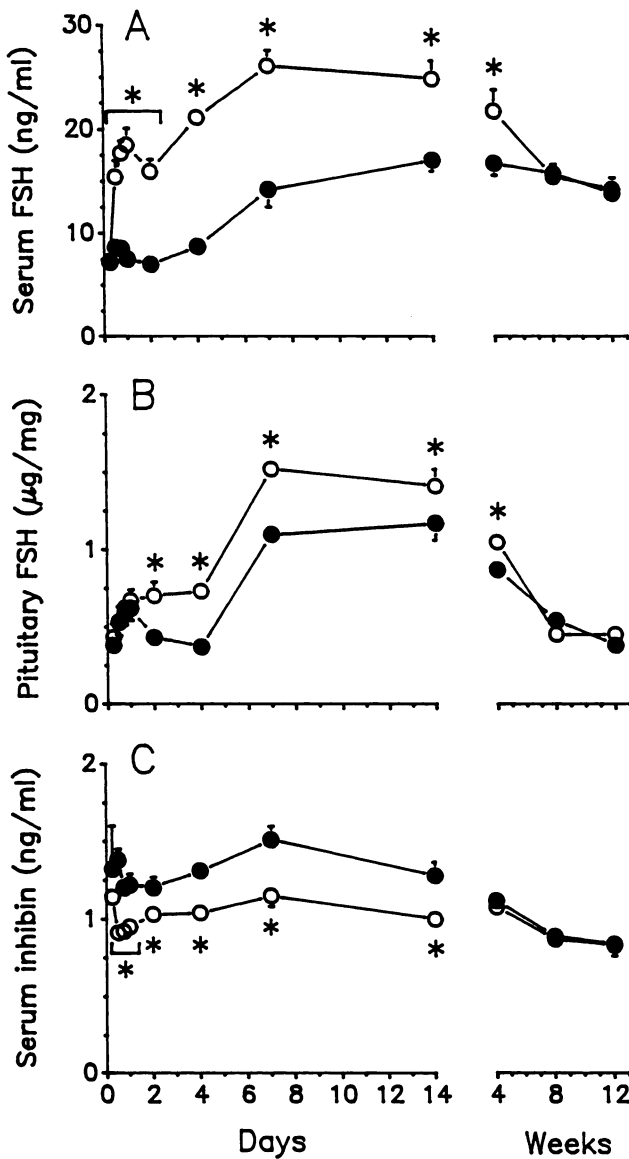


FIG. 4. Effects of hemicastration on concentrations of serum FSH (A), pituitary FSH (B), and serum inhibin- α (C) in hemicastrated (open circles) and intact (filled circles) prepubertal (14 days old) male rats. Values are presented as the mean \pm SEM. For points with no SEM bars, the values fell within the symbols. *Differs from intact control ($P < 0.05$).

bertal and adult rats (Cunningham et al, 1978; Frankel and Wright, 1982; Rivier et al, 1989). We observed, however, that the rise in serum FSH was greater in prepubertal than mature animals and that coincident increases in the concentrations of pituitary FSH were apparent only in prepubertal males. These observations suggest that there may be temporal, age-related changes in the ability of the pituitary gland to increase FSH synthesis following hemicastration, and they are consistent with reports of age-related decreases in the rise of FSH following bilateral castration (Hermans et

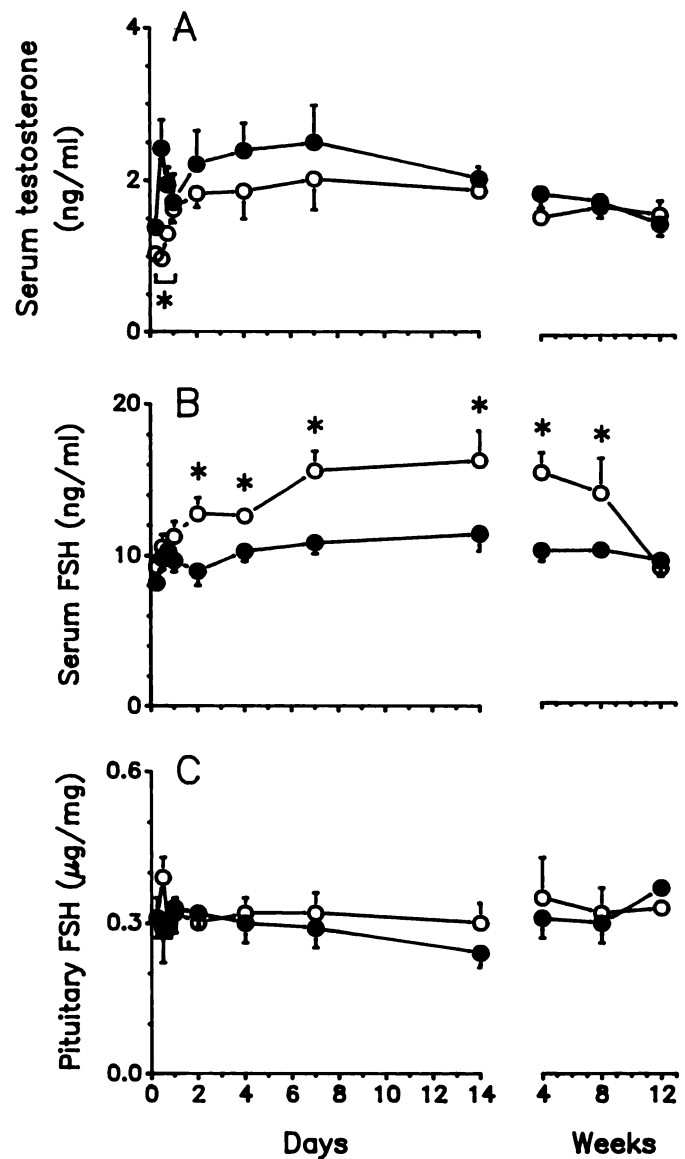


FIG. 5. Effects of hemicastration on concentrations of serum testosterone (A), FSH (B), and pituitary FSH (C) in hemicastrated (open circles) and intact (filled circles) mature (10 to 12 months old) male rats. Values are presented as the mean \pm SEM. For points with no SEM bars, the values fell within the symbols. *Differs from intact control ($P < 0.05$).

al, 1980). It is unfortunate that serum FSH was not evaluated in the study of Johnson and Neaves (1981), as this information may have helped us interpret the role of FSH in stimulating testicular growth in aged animals.

The exact mechanisms controlling FSH secretion in male rats are not well understood but may be due to a reduction in testicular inhibin, a hormone implicated in regulating the secretion of this gonadotropin (Scott and Burger, 1981; de Jong and Robertson, 1985). Although injections of inhibin-containing preparations have been shown to reduce serum

FSH in both adult and prepubertal male rats (Hermans et al, 1980; Lorenzen et al, 1981), this hormone is now considered a more important regulator of FSH secretion in immature animals (Hermans et al, 1980; Rivier et al, 1988). For example, removal of endogenous inhibin by passive immunization was shown to increase plasma FSH levels in male rats during the first 21 days of age but not at later ages (Rivier et al, 1988). This hypothesis is supported by the current data, since an increase in serum FSH accompanied by a fall in inhibin- α levels was observed only in hemicastrated prepubertal rats. Previously, Rivier et al (1989) reported that serum FSH and inhibin- α concentrations were inversely related when measured over the 7 days following hemicastration in prepubertal (15 days old) but not pubertal (45 days old) rats. We have extended their observations to demonstrate that the return of serum inhibin- α concentrations to intact levels in prepubertal rats precedes the fall in serum FSH. These results strengthen the hypothesis that a decrease in inhibin precipitates the hemicastration-induced increase in FSH secretion in young animals.

In the adult rat, the most important regulator of FSH secretion may be testosterone rather than inhibin (Summerville and Schwartz, 1981). Indeed, it has been suggested that testosterone alone accounts for the testicular feedback signal in adult rats and that inhibin acts as a secondary modulator that is superimposed on the steroid feedback mechanism (Jones et al, 1985). Rivier et al (1989) further demonstrated that exogenously administered testosterone blocked the stimulatory effect of hemicastration on FSH secretion in both prepubertal and pubertal rats. However, it seems unlikely that the short-term decrease (1 day) in testosterone secretion observed following hemicastration in this study was capable of causing the extended rise in FSH levels. Instead, alternative mechanisms should be considered. For example, other factors present in gonadal tissues such as follistatin (Esch et al, 1987) and a Sertoli cell steroid (3 α -hydroxy-4-pregnen-20-one; Wood and Wiebe, 1989) also have been shown to selectively suppress FSH secretion. Removal of these or other as yet unidentified factors may be more important regulators of FSH secretion following the removal of one testis in the adult rat.

In all hemicastrated rats, a compensation in testosterone secretion was observed within 1 day after surgery. The exact mechanisms responsible for this compensation are still unclear. Consistent changes in LH secretion following hemicastration have not been observed (Frankel and Mock, 1982; Frankel and Wright, 1982; current study), nor did we observe any changes in pituitary LH concentrations. In intact rats, elevated concentrations of plasma FSH are positively correlated with increasing testicular LH receptor concentrations and testosterone production from 15 to 40 days of age (Ketelslegers et al, 1978). Therefore, we speculated that the increase in serum FSH after hemicastration

might be similarly affecting testicular sensitivity via changes in gonadotropin receptors. We did observe a gradual increase in LH receptors with increasing age in prepubertal rats; however, the receptor concentrations were similar between hemicastrated and intact animals in both the prepubertal and mature animals. Frankel et al (1984) also reported that LH receptor concentrations were unaffected when examined at one time point (24 hours) after hemicastration in 4- to 5-month-old rats. Instead, we observed an increase in LH receptor content in prepubertal hemicastrated rats that apparently was related to the alteration in testis size.

Leydig cell numbers do not change in prepubertal or adult rat testes following hemicastration, and increased Leydig cell volume in the remaining testis has only been identified in adults (Bergh et al, 1982; Furuya, 1990). Thus, the increased receptor content in prepubertal rats was likely due to an increase in the number of binding sites per cell. This may account, in part, for the observed compensation in androgen secretion in these animals. However, it is doubtful that this is the major factor since androgen compensation had occurred by day 1, and an increase in testicular receptor content was not observed until day 7. Neither LH receptor concentrations nor contents were altered by hemicastration in adult rats, yet testosterone levels in hemicastrated animals returned to intact levels by day 1. Testicular FSH receptor concentrations declined with age as described previously (Ketelslegers et al, 1978) and were similar between hemicastrated and intact animals. However, there was a slight increase in FSH receptor content in prepubertal HC rats related to the increase in testis size. This testicular hypertrophy has been reported to be due to an increase in Sertoli cell size rather than number (Putra and Blackshaw, 1982). Thus, the increased FSH receptor content in testes of hemicastrated rats in the current study was likely due to an increase in numbers of receptors per cell.

Similar studies conducted in sheep (Brown et al, 1987, 1988) and cattle (Schanbacher et al, 1987) also reported no significant changes in gonadotropin receptor concentrations but increased receptor contents and testis size after hemicastration of prepubertal animals. These data suggest that compensatory androgen secretion, observed within hours after hemicastration, is not related to an increase in the sensitivity of the remaining gonad to circulating gonadotropins. Instead, there is evidence that this response may be neurally mediated. Frankel et al (1984) showed a partial block of the hemicastration response by hemivasectomy contralateral to the excised testis, suggesting that the inferior spermatic nerves, which innervate the epididymides and vasa deferentia, may be involved. Furthermore, Moger and Anakwe (1986) demonstrated that compensatory androgen secretion was prevented by intra-testicular injection of DL-propranolol (a β -adrenergic receptor antagonist). Taken

together, these data suggest that removal of one testis may activate adrenergic neurons innervating the testis and, through β -receptors, stimulate testosterone secretion (Moger and Anakwe, 1986).

Acknowledgments

The authors thank NIDDK for the rat LH and FSH RIA kits and the ovine (NIADDK-FSH-S16) used in the radioreceptor assays. We also thank J.F. Duncan, Jr. (Duncroft, Inc., Lovettsville, VA) and M.N. Flora (USUHS) for assistance with inhibin conjugation and antibody production.

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