Effects of Exogenous Testosterone on Testicular Luteinizing Hormone and Follicle-Stimulating Hormone Receptors During Aging

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During aging, the male Japanese quail exhibits a loss of fertility, increased morphological abnormalities in the testes, and a higher incidence of Sertoli cell tumors. Although there is a coincident loss of reproductive behavior, plasma androgen levels remain high until testicular regression occurs in association with senescence. The purpose of this study was to compare mean specific binding of chicken luteinizing hormone (LH) and follicle-stimulating hormone (FSH) as a measure of testicular receptors during identified stages during aging. Males were categorized according to age (young = 9 months, middle aged = 24 months, or old = 36+ months) and sexual behavior (active or inactive). Testicular samples were collected immediately after perfusion with 4% paraformaldehyde from the following groups: young active (n = 8), young photoregressed (n = 5), young photoregressed plus testosterone implant (n = 4), middle-aged active (n = 8), middle-aged inactive (n = 4), old inactive (n = 5), and old inactive plus testosterone implant (n = 6). A crude plasma membrane fraction was prepared from the testes of each bird and an aliquot deriving from 10 mg of testicular tissue was used for binding assay. Specific binding of labeled LH or FSH was expressed as percentage of total radioactive hormone. Results showed significant ($P < 0.05$) age-related decreases in both FSH and LH receptor numbers. The highest FSH binding was found in young and middle-aged active males, with low binding in old inactive males. Testicular LH binding decreased during aging, with a sharp decrease in middle-aged males, which was similar to old males. Testosterone implants weakly stimulated FSH and LH binding in old males. Both LH and FSH binding decreased in photoregressed young males. However, testosterone implants stimulated increased LH binding, but did not affect FSH binding in young photoregressed males. These results provide evidence for separate regulation of testicular LH and FSH receptors, with testosterone stimulation of LH receptor, but not FSH receptor number in young males. However, during aging there appears to be a loss of this response, potentially because of the reduced efficacy of testosterone stimulation, thereby implying a diminished capacity for response with aging. Exp Biol Med 227:830–836, 2002

Key words: reproductive aging; testes; luteinizing hormone receptors; follicle-stimulating hormone receptors; Japanese quail.

There is considerable debate whether endocrine events that contribute to the age-related loss of reproduction and whether altered testicular or hypothalamic function are the primary causes of reproductive senescence. It is clear that there is an age-related decline in spermatogenesis and fertility, which may be due to declining function of the entire reproductive axis (1–3). A number of studies have focused on Leydig cell function during aging as a primary cause for declining reproductive capability. These studies have shown decreased Leydig cell function and number in addition to luteinizing hormone (LH) receptors; however, the reason for this age-related change is not clear (4–8). In mammals, there is debate about the role of follicle-stimulating hormone (FSH) in male fertility (9, 10). Expression of the FSH receptor varies with the stages of the seminiferous epithelium and is downregulated by elevated FSH levels (11, 12). The FSH receptor knockout mouse shows diminished spermatogenesis and fertility (13). Interestingly, FSH β–subunit–deficient male mice remained fertile but had reduced testis size (14).

The hypothalamic-pituitary-gonadal (HPG) axis in the male Japanese quail operates in a similar manner to that in mammals. LH and FSH are produced by the pituitary gland under the control of gonadotropin-releasing hormone-I (GnRH-I). Gonadal steroids regulate GnRH-I and pituitary gland release of gonadotropins. The role of FSH in testicular function has been described in male quail and in other birds (15–17). The regulation of testes function by gonadotropins in Japanese quail is similar to mechanisms found in mammals (3, 18). Because the Japanese quail is a seasonal

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breeder, these birds use photoperiodic cues or long photoperiods in timing reproductive function. Conversely, photoregression occurs when birds experience diminished day length, especially fewer than 12 hr of light. Within 1 to 2 weeks of short days, the birds molt, spermatogenesis declines, and the testes and cloacal gland regress. The signal for photoregression is interpreted from input to the central nervous system, resulting in a sharp drop in GnRH-I release and a loss of gonadotropin production and testicular regression. Over the years, we have observed that the process of aging appears to be a much more gradual loss in reproduction, which occurs despite plasma levels of LH, FSH, and androgen that are characteristic of reproductive adults (18). Therefore, these appear to be very different physiological responses, which both result in eventual loss of reproductive function.

We have been examining elements of the HPG axis during the process of aging in the Japanese quail model. Our studies in male quail have shown an age-related loss of fertility and cessation of sexual behavior, which precede measurable changes in plasma steroids, LH, or FSH (3, 8, 18–20). Furthermore, plasma androgen levels decreased significantly only in old, sexually inactive (senescent) individuals in whom the testes have regressed (3, 19). Dispersed testicular preparations made from males of varied age and reproductive status revealed continued Leydig cell production of testosterone *in vitro,* even in old males (3). This was despite increasing morphological abnormalities observed in aging male quail and a higher incidence of Sertoli cell tumors (3, 21, 22). In addition, plasma LH remained relatively constant throughout aging, whereas plasma FSH increased in old sexually inactive males (3). The increase in plasma FSH has been observed over a number of species, including mammals (20). From these data, it became apparent that gonadotropin receptors play a key role in the age-related alterations in testicular function. Therefore, the purpose of this experiment was to study relative changes in both LH and FSH receptors during aging and to compare these responses with photocastrated males and young or old males given exogenous testosterone.

Materials and Methods

Experimental Animals. The Japanese quail (*Coturnix japonica*) were hatched from eggs collected from a random-bred white egg strain. In this strain, chicks hatch after 17 days of incubation and the birds mature sexually in 6–8 weeks. Birds were provided feed (Purina Game Bird Startina or Layena) and water *ad libitum.* Birds were brooded and housed in mixed sex groups in controlled environment (15-hr light:9-hr dark cycle) and maintained according to NIH Assurance Statement (A3270-01) and UMCP IACUC approval. Because this was a cross-sectional experiment, males were categorized according to age as young (9 months), middle-aged (2 years), and old (3+ years) and sexual behavior (active or inactive). A subset of young birds was placed on a short photoperiod (8-hr light:16-hr dark) in a separate environmentally controlled room. Based on previous experiments, male quail fully regress within 3 weeks and plasma androgen levels become very low (50 pg/ml). In this experiment, young males were placed on short days and remained there for a month. At the end of this time, males were tested for sexual behavior; none were behaviorally active. In addition, the cloacal gland, which is androgen dependent, was checked for function. All males had reduced cloacal gland area and no foam production, which also verified photocastration. Males in the other groups were sexually experienced; several months before the experiment they were housed individually. All males were tested behaviorally to assess reproductive status in a behavioral test (23). This allows separation of males that are the same age, but reproductively active or senescent (3).

Testosterone Implants. Testosterone implants were silastic tubing (Dow Corning 602-265: OD = 0.95 mm; ID = 0.26 mm; Corning Inc., Corning, NY) filled with crystalline testosterone (Steraloids, Inc, CA). The length of implant filled with hormone was 20 mm, and the ends were sealed with silastic implant sealer (Dow Corning, Corning, NY). Implants were rinsed in methanol, checked for leaks, and soaked in physiological saline overnight before implant. Implants were inserted through a 16-gauge syringe needle using a plunger and placed under the skin on the back of the neck, with a suture to close the skin, if necessary. One implant was administered per treatment bird. Previous use of these implants in our birds has shown that this size implant results in physiological levels of plasma testosterone (20).

Radioreceptor Assays. LH or FSH receptors were determined by specific binding of labeled LH or FSH to a plasma membrane fraction prepared from 10 mg of testicular tissue. LH and FSH were labeled with ^{125}I (Amersham, Horsham, PA) by using the lactoperoxidase method. Specific radioactivity of labeled LH and FSH was about 25,00 cpm/ng and 40,000 cpm/ng, respectively. Testes were collected immediately following perfusion with 4% paraformaldehyde. Testicular tissues fixed with 4% paraformaldehyde were stored at −80°C. Membrane preparations were prepared after homogenization and centrifugation according to a modification of the method of Kikuchi and Ishii (25), which is validated for Japanese quail tissue. As detailed below, the method using fixed tissue was validated compared to the use of fresh frozen tissue. The binding reactions were performed at 37°C and 1.5 hr for LH and 2.5 hr for FSH. An excess amount of glycoprotein prepared from quail pituitaries was added to a reaction mixture for detection of nonspecific binding. Binding was expressed as per unit tissue weight or an estimate was calculated for percent bound/testis.

Because perfused tissue was used for these assays, a comparison was made to a fresh-frozen sample taken from young males. Specific binding for LH receptor was 5.65% in the fresh-frozen testis sample compared to experimental samples, which ranged from 1.09 to 5.65%. Specific bind-

ing for FSH receptor was 18.98% in the fresh-frozen sample compared with a range of 13.13 to 19.84% in experimental samples taken from young males. In addition, we have examined the morphology of testes during the process of aging to determine the loss of cell types. These data have shown that the Sertoli cell number does not change during aging, even when the testes become regressed (22). In a separate study, Leydig cell number and *in vitro* response to LH challenge were conducted to compare young reproductive and aging males. Results showed that both Leydig cell number and ability to release testosterone did not decline in regressed testicular tissue. Based on these results, we expressed receptor number according to per mg tissue as data on the concentration of receptor in the tissue and we also estimated total receptor number to give information on total gonadal content. In this way, receptor number was evaluated in a constant amount of tissue and then as an estimate of the total receptor number per testis at each age and reproductive state.

Experimental Design and Sample Collection. Body weight and cloacal gland area were measured. Sexual behavior was assessed in a 5-min behavioral test in which a receptive female was introduced into the male's cage. Courtship and mating behaviors were recorded; males were tested on 3 successive days (23). There were three additional groups of males: young photoregressed males, young photoregressed males with a testosterone implant, and old senescent males with a testosterone implant. All testosterone-implanted males showed restored courtship and mating behavior, which was qualitatively and quantitatively similar to that observed in younger males. Testicular samples were collected from Japanese quail (Coturnix japonica) immediately after perfusion of the bird with 4% paraformaldehyde. Gonads were then post-fixed in 4% paraformaldehyde.

Samples were collected from birds of the following ages and treatments: young active (n = 8), young photoregressed (n = 5), young photoregressed plus testosterone implant (n = 4), middle-aged active (n = 8), middle-aged inactive (n = 4), old inactive (n = 5), and old inactive plus testosterone implant (n = 6). The design was randomized block and data were analyzed by analysis of variance followed by multiple range tests.

Results

Body weight did not change significantly during aging (Fig. 1). However, mean testes weight decreased significantly ($P < 0.05$) during aging and in both behaviorally active and inactive males (Fig. 2). Average paired testis weight varied from 3.9 ± 0.3 g to 0.15 and 0.19 ± 0.05 g in young males that were active, photoregressed, or photoregressed plus testosterone, respectively. Middle-aged males had testes weights of 0.92 ± 0.3 g in active birds and 0.28 ± 0.15 g for inactive males. Old inactive males had fully regressed testes that weighed 0.25 ± 0.1 g; testosterone treatment resulted in a slight increase to 0.32 ± 0.2 g. Although these testes weight differed greatly, the assay was

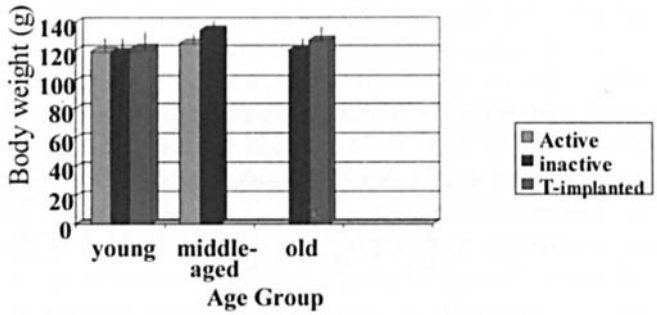

Figure 1. Body weight of males with age and reproductive status. No significant changes were found.

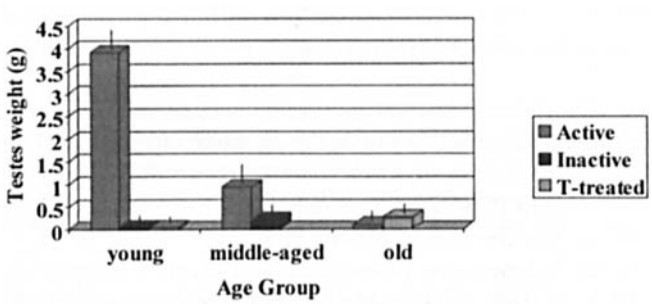

Figure 2. Testes weight in males of different ages and reproductive status. There was a significant ($P < 0.05$) decrease during aging and in the photoregressed young males.

conducted for samples from each male, with 10 mg of tissue used per assay. As such, testes size did not pose an issue for single sample assays. Cloacal gland area also decreased during aging, with a significant ($P < 0.05$) decrease in young photoregressed, middle-aged and old behaviorally inactive males (Fig. 3). Specific binding of LH (%/10 mg tissue) decreased during aging but showed significant ($P < 0.05$) decrease in middle aged active males compared with young active males (Fig. 4). Specific binding of LH increased significantly ($P < 0.05$) in young photoregressed males given testosterone implants (Fig. 4). A similar significant ($P < 0.05$) stimulation of LH receptor number was observed in old senescent males given a testosterone implant; however, the magnitude of response was not as great (Fig. 4). To examine LH receptor binding relative to the entire testis volume, the specific binding in 10 mg of tissue was multiplied by testis weight (Fig. 5). This allowed com-

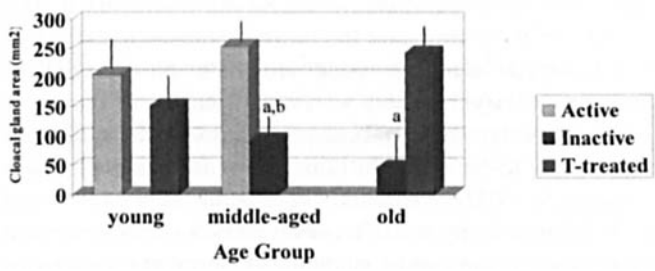

Figure 3. Cloacal gland area (mm²) in males during aging and with reproductive status. There were significant decreases ($P < 0.05$; letters denote significant differences with group) during aging in the young photoregressed, middle-aged, and old inactive males.

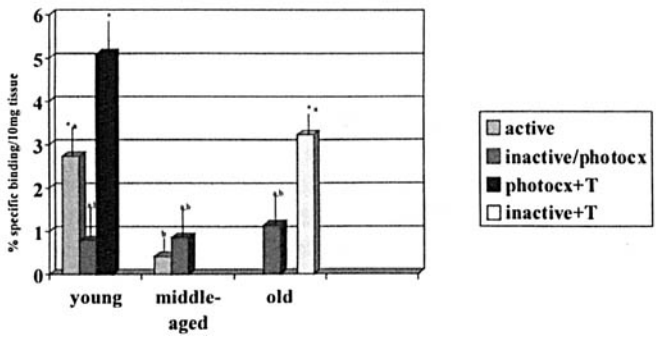

Figure 4. Specific binding of LH (%/10 mg tissue) decreased in middle-aged males, with old senescent males aging and with reproductive status. There was a significant ($P < 0.05$) increase (*) in young photoregressed and old inactive males given testosterone implants. Age-related significance depicted by letters a and b; only the middle-aged active males showed a decrease.

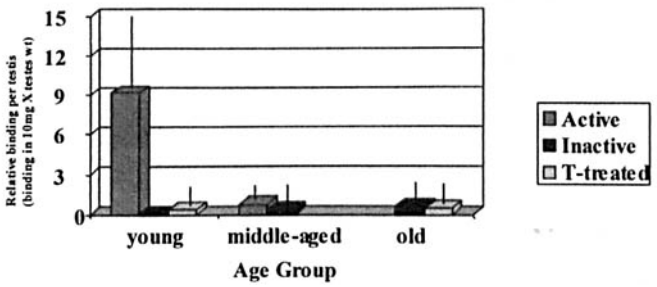

Figure 5. Relative binding of LH estimated for the entire testis (% specific binding/10 mg tissue × testis weight) during aging and with reproductive status. The significant ($P < 0.05$) decrease during aging may be clearly seen when testis volume is considered.

parison of an estimate of relative receptor number per testis across males that had fully function versus regressed testes. When expressed as relative receptor number/testis, the LH receptors decreased significantly ($P < 0.05$) during aging (Fig. 5).

Specific binding of FSH (%/10 mg tissue) decreased significantly ($P < 0.05$) during aging, with middle-aged inactive males showing transitory decline (Fig. 6). Testosterone implants had no effect in photoregressed young males and had some effect in senescent, testosterone implanted males (Fig. 6).

This decrease was also observed when binding was expressed as the relative receptor number estimated for the entire testis (Fig. 7).

Discussion

Comparison of Reproductive and Photoregressed Young Quail. This study deals with two endocrine processes, photoperiodic regulation of reproduction and reproductive aging. During long days, hypothalamic stimulation of the HPG axis results in activation of the reproductive axis, including testicular function. Both LH and FSH receptor numbers reflect reproductive activation and support spermatogenesis and steroidogenesis. Conversely, short days shut down the reproductive system in quail, resulting in a loss of hypothalamic stimulation of the

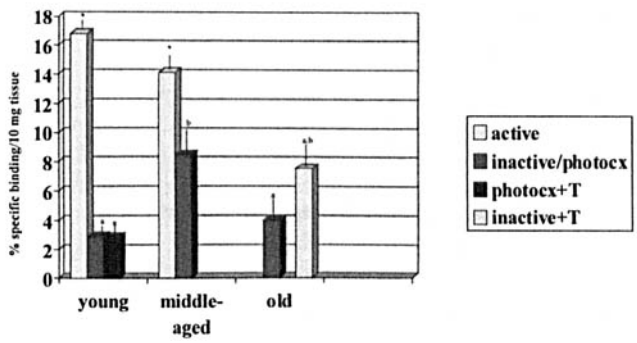

Figure 6. Specific binding of FSH (%/10 mg tissue) decreased significantly ($P < 0.05$) during aging, with middle-aged inactive males intermediate in receptor number. Young and middle-aged active males were significantly higher ($P < 0.05$; *) than other groups. Photocastration resulted in significantly ($P < 0.05$) reduced receptor number, which was not affected significantly by testosterone treatment. Age-related significance among groups depicted by letters a and b; young and middle-aged active males were more significant ($P < 0.05$) than all other groups.

HPG axis and photocastration. Because the HPG axis is essential turned off, the photocastrated male has low levels of plasma steroids and the testes regress. In previous studies in which male quail were photoregressed, plasma androgen levels averaged about 50 pg/ml plasma associated with testicular regression (24). Therefore, in the photoregressed male quail, the inhibition of the system originates at the level of the hypothalamus, with reduced GnRH-I stimulation of the pituitary gonadotropins and resulting testicular regression. Because the primary site of the testicular regression is the seminiferous tubules, there is a loss in the volume and weight of the testis (25). In addition, the loss of gonadotropin stimulation of the Leydig cells results in diminished androgen production, leading to regression of the cloacal gland, which is androgen dependent. Therefore, the loss of plasma androgen is further verified by the shrinkage and loss of function by the cloacal gland, which was decreased in young photoregressed males.

As mentioned above, the testes retain the Leydig and Sertoli cells, even with photoregression so that *in vitro* challenge still induces testosterone release (Ottinger, Hargrove, and Thompson, unpublished data). Our data are in agreement with previous observations that the adult reproductive quail has a high testicular FSH receptor number (16). Those data also indicated a role for testosterone in stimulating FSH receptors. Therefore, it would be expected that a loss of FSH receptors would occur in the photocastrated males, which indeed showed a sharp decline in FSH receptors. This verifies the role of the HPG axis in maintaining the FSH receptors via the HPG axis. However, there was no change in FSH receptor number in response to exogenous testosterone in young photocastrated males. This suggests that testosterone may not be the critical element modulating FSH receptor numbers.

It therefore appears that there is a fundamental difference in the photoregressed condition as compared with the aging male. Furthermore, the middle-aged animals are the most difficult to explain because it is not clear whether they

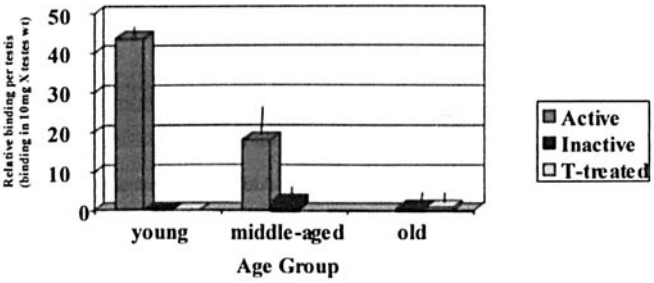

Figure 7. Relative binding of FSH estimated for the entire testis (% specific binding/10 mg tissue × testis weight) during aging. There was a significant ($P < 0.05$) age-related decrease in binding during reproductive aging.

are becoming photorefractory or simply going through the initial stages of reproductive aging. There are two reasons for which we hypothesize this difference between aging and photorefracotoriness. First, photoregression relies on hypothalamic regulation of the reproductive axis, which means decreased GnRH-I–stimulated pituitary gland gonadotropin production and release. As a result, testicular function declines and spermatogenies ceases. During aging, there is a more gradual decline in hypothalamic GnRH-I stimulation of the pituitary gland. Moreover, there is no discernible loss in plasma LH levels, even in old senescent males. Specifically, our data have shown little if any decline in plasma LH and only eventual increasing plasma FSH in the very oldest animals. Furthermore, plasma androgen levels do not significantly decrease until after the male has become fully regressed and senescent. As such, there is a fundamental difference in the hormonal support of the testes in photocastrated versus aging males. Second, the response of the young photoregressed male testis to testosterone replacement was dramatic for the LH receptors, which were induced by the steroid treatment. Although testosterone treatment induced LH receptors in both young photocastrated and old regressed males, there was a quantitative difference in the response in that there was a 550% increase in LH receptors in young photocastrated compared with young photocastrated plus testosterone. Further, when this comparison was made in old males, there was a 280% increase in LH receptors in old regressed males that received exogenous testosterone compared with old senescent males. This illustrates the more muted response in the old male compared with the young animal. Therefore, our data argue that the middle-aged male shows initial stages of the process of aging

Relationship of Aging to Reproductive Function. In regard to reproductive aging, males in our study were categorized according to chronological age as well as sexual behavior; the reproductive status of males in a group should be relatively similar. It is clear that the middle-aged male is in a transitional state. Old males did show some response to testosterone, with a moderate increase in FSH receptor number. However, overall FSH receptors did not appear to respond directly to testosterone, implying that other hormones or factors are critical for stimulation of FSH receptor expression.

Conversely, our data provide strong supporting evidence for steroid hormone stimulation of LH receptors. This was clear in the sharp decrease in LH receptors in photocastrated males, in which circulating testosterone levels are very low. Similar decrease in LH receptors was reported in photorefractory males of the white-crowned sparrow, in which testicular testosterone production *in vitro* was undetectable (26). Furthermore, in the present study, exogenous testosterone resulted in much greater specific binding of LH, indicating expression of high LH receptor number. As the males aged, the number of LH receptors decreased associated with decreased testes weight. Separate experiments have shown that plasma androgen levels decrease during aging (18, 19). However, this decrease in plasma androgen levels was gradual during aging and occurred after loss of sexual behavior. As such, the decline in LH receptors in aging males appears to be associated with loss of plasma testosterone. It is at this stage in aging that we have also observed decreasing hypothalamic response relative to the GnRH-I system and regulatory neurotransmitter and neuropeptide systems (27). As such, it appears that testicular LH receptor number is at least partially dependent on testosterone stimulation. This is further supported by the strong response to testosterone in old senescent males. Therefore, testosterone appears to be a critical factor in the regulation of LH receptor number in the testis.

Finally, morphological changes during reproductive aging add additional insight. As mentioned earlier, there is increased incidence of morphological abnormalities or pathologies, including lesions and tumors (22). In addition, there is increased deposition of lipofusion, which may signal loss of cellular function (3). Interestingly, despite testicular regression, Leydig's cells remain relatively unchanged in morphological appearance. It is the increasing disorganization and loss of spermatogenesis in the seminiferous tubules that contributes to the physical regression of the testis. This observation may provide an explanation for the apparent contradictory results, specifically that plasma androgen levels continue to remain high while at the same time, the testes are regressing in aging males. Furthermore, it appears that gonadotropin support either continues at constant levels or increases in the case of FSH. Therefore, it would appear that gonadotropin receptors must be at least partially responsible for the loss of testicular function. In an earlier experiment, we examined FSH receptor number and binding affinity during aging (Ottinger *et al.*, unpublished data). These results showed that there was a loss of FSH receptors during aging when expressed as number per testis, rather than per weight. When expressed on a per weight or protein basis, the number decreased relatively less due to the loss of seminiferous tubules accompanying testicular regression. Furthermore, the traditional view has been that testosterone is necessary for the stimulation of Sertoli's cells and therefore essential for function of these cells.

There are similarities in our data in quail with the literature in mammals. There is evidence for a gradual decline in testicular function during aging (1, 2). Studies have shown that plasma levels of androgen do eventually decline in mammals; however, a measurable decrease in plasma steroid hormones usually occurs after a loss in fertility. It is not clear whether the endocrine basis for the loss in fertility and plasma steroid hormones resides in the declining function of the entire HPG axis or if Leydig and Sertoli cell function are to blame. Evidence from humans indicates that the function and possibly the number of Leydig's and Sertoli's cells decrease in senescent individuals (2, 28–30). Other studies have shown an age-related decline in unbound plasma testosterone, which was associated with elevated LH and FSH levels and decreased inhibin. Because there are many other factors that must be considered in humans, it has been difficult to develop a clear model of the process of reproductive aging (3).

Studies in the rat provide more insight into the process of male aging. These studies have shown that Leydig cell function declines during aging, resulting in reduced testosterone production *in vivo* and *in vitro* (7, 8, 29–31). The decline in testosterone production was not associated with any measurable change in Leydig cell number (29, 30). Moreover, Leydig's cells retained an ability to respond *in vitro*, indicating that steroidogenesis became impaired in older animals. This may be due in part to reduced mobilization of cholesteryl ester in response to HCG found in Leydig's cells from old rats (8). A similar age related alteration in testosterone synthesis was observed in Fisher 344 rats, in which plasma testosterone and testicular LH receptors decreased during aging (4). Overall, aging Leydig's cells appear to loose steroidogenic function, despite retaining responsiveness to stimulation. Old and young rats subjected to chemical ablation of Leydig's cells from ethane dimethanesulfonate showed the ability to produce new Leydig's cells (30, 31). Moreover, plasma testosterone levels in old males were similar to those of young animals, implying that the indigenous Leydig's cells become dysfunctional with aging. However, it is not clear whether this occurs because of loss of receptors or to some other factor.

In conclusion, specific binding of LH to testicular receptors decreased during aging. Exogenous testosterone restored LH receptors in senescent males and enhanced LH receptor number in young photocastrated males. There was a similar age-related decline in testicular FSH receptors. Photoregression was associated with decreased testicular FSH receptor number. However, exogenous testosterone did not consistently alter FSH receptor concentration. Therefore, there appear to be distinct differences in the age related pattern in testicular LH and FSH receptor number and differential regulation of these testicular receptors by testosterone.

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