PERGAMON

Food and Chemical Toxicology 40 (2002) 1815–1825

Food and Chemical Toxicology

www.elsevier.com/locate/foodchemtox

Lack of effects of oxolinic acid on spermatogenesis in young adult and aged Wistar rats

M. Yoshida[a,*], T. Kitani[a], A. Takenaka[a], K. Kudoh[a], S-I Katsuda[a], K. Taya[b], Y. Kurokawa[a], A. Maekawa[a]

[a]*Department of Pathology, Sasaki Institute, 2-2, Kanda-Surugadai, Chiyoda-ku, Tokyo 101-0062, Japan*
[b]*Veterinary Physiology, Tokyo University of Agriculture and Technology, 3-5-8, Saiwai-cho, Fuchu-chi, Tokyo 183-8509, Japan*

Accepted 15 February 2002

Abstract

Prolonged treatment with oxolinic acid is known to elevate serum luteinizing hormone (LH) levels, resulting in induction of Leydig cell tumors in rats. In a carcinogenicity study of the compound, tubular atrophy of the testis was also increased, suggesting that oxolinic acid might affect spermatogenesis. The present study was therefore performed using rats of different ages with a particular focus on seminiferous tubule alteration and its relation to Leydig cell proliferation. Young adult (7 weeks of age) and aged (52 weeks of age) males of the Wistar strain were administered oxolinic acid at dietary concentrations of 0 (basal diet), 300, 1000 or 3000 ppm for 4 (all groups), 13 (0 and 3000 ppm groups), 26 (0 and 3000 ppm groups), or 52 weeks (0 and 3000 ppm groups of aged rats). Serum LH levels were elevated in both young adult and aged animals treated with 3000 ppm at most examined time points. While testosterone levels were also increased at the early time points in young adult, this was not the case in older animals. Elevation of the incidences of foci and/or focal hyperplasia of Leydig cells was noted but was only slight limited to aged rats treated with 3000 ppm after 26 weeks. Furthermore, it did not appear to be related to seminiferous tubular alteration. No treatment-related histopathological abnormalities could be detected in any treatment group, and morphometrical stage analysis of spermatogenesis conducted for the control and 3000 ppm-treated groups demonstrated no lesions. These results provide strong evidence that prolonged oxolinic treatment does not directly induce testicular toxicity or altered spermatogenesis in either young adult or aged rats, except for slight increase of Leydig cell proliferative lesions caused by elevated serum LH levels. Aged rats might have higher sensitivity than young adults to the effects of oxolinic acid on proliferative lesions of Leydig cells.
© 2002 Elsevier Science Ltd. All rights reserved.

Keywords: Oxolinic acid; Spermatogenesis; Leydig cell; Tubular atrophy; Rat.

1. Introduction

Oxolinic acid (1-ethyl-1,4-dihydro-6,7-methylenedioxy-4-oxo-3- quinolinecarboxylic acid) is a first-generation quinoline derivative compound with antimicrobial activity, used for clinical treatment of urinary tract bacterial infections (Gleckman et al., 1979). In recent decades, this compound has also been employed in the agricultural field for prevention and cure of plant diseases caused by bacteria (Hikichi et al., 1989), and may thus be present in agricultural products. In a carcinogenicity study, oxolinic acid was found to significantly increase the incidence of Leydig cell hyperplasia and/or tumors and tubular atrophy in the testes of male Wistar rats given dietary concentration of 1000 ppm, although no carcinogenicity was evident in CD-1 mice (Yamada et al., 1994a). A DNA repair assay of the compound using *Escherichia coli* and an Ames test using *Salmonella* test strain TA102 showed positive findings; however, its mutagenicity was considered to be due to antimicrobial activity rather than direct damage to DNA (Cozzarelli, 1980; Osheroff et al., 1983). The available data thus indicate the possibility that oxolinic acid does not induce Leydig cell tumors by direct action at the DNA level (Yamada et al., 1994a).

The function of Leydig cells is regulated by the hypothalamus–pituitary–gonadal control system, mainly by luteinizing hormone (LH) released from the pituitary.

Abbreviations: LH; luteinizing hormone; RIA; radioimmunoassay

* Corresponding author. Tel.: +81-3-3294-3286; fax: +81-3-3294-3290.

0278-6915/02/$ - see front matter © 2002 Elsevier Science Ltd. All rights reserved.
PII: S0278-6915(02)00168-0

Prolonged elevation of serum LH levels is known to induce Leydig-cell proliferative changes in rats (Chatani et al., 1990, 1996; Hosokawa et al., 1993). In a previous study, long-term treatment with 1000 or 3000 ppm oxolinic acid caused increase of serum LH level and the increase was more obvious in aged than in young adult rats (Yamada et al., 1994b). In mechanistic studies of the development of Leydig cell tumors in rats caused by oxolinic acid, Yamada et al. indicated that the treatment modulated the dopamigenic system in the hypothalamic–pituitary axis, resulting in increases of the serum LH level (Yamada et al., 1994b, 1995a).

In the previous carcinogenicity study, the incidence of tubular atrophy in the testis was increased (Yamada et al., 1994a). Testicular tubular atrophy is known to be the most common lesion observed in rats given various chemicals or in aged rats. In the latter cases, atrophic tubules were often replaced by developing large Leydig cell tumors. The previous carcinogenicity study did not elucidated whether tubular atrophy in the high-dose group was related to tumor development, or if there was the potential for direct toxic effects of oxolinic acid on the rat testicular epithelium.

The present study was conducted (1) to investigate whether oxolinic acid exerts direct effects on the spermatogenesis or the seminiferous tubular epithelium in rats and (2) to assess any relationship between tubular atrophy and development of proliferative lesions of Leydig cells associated with oxolinic acid treatment. In addition, differences in sensitivity between young adult and aged animals were examined.

2. Materials and methods

2.1. Chemicals

Oxolinic acid (1-ethyl-1,4-dihydro-6,7-methylenedioxy-4-oxo-3-quinoline carboxylic acid, 93.8% purity) was supplied by Tanabe Co. Ltd (Osaka Japan).

2.2. Animals and housing

Young adult and aged male Wistar rats were purchased from CLEA Japan (Tokyo, Japan). This strain was selected because it was used in the previous carcinogenicity study (Yamada et al., 1994a). After acclimatization to the laboratory environment, animals at 7 weeks and 52 weeks of age were provided for the studies. They were housed in plastic cages (one or two animals/cage) with hardwood chips for bedding and maintained under conditions of controlled temperature (24±2 °C), humidity (55±10%) and lighting (12-h light/dark cycle). Drinking water was available ad lib. Animal care and use followed the NIH Guide for the Care and Use of Laboratory Animals.

2.3. Diet preparation

A set amount of oxolinic acid was mixed with a portion of the powdered basal diet (CRF-1, Oriental Yeast Co. Ltd, Tokyo, Japan) using a pestle to prepare a premixture which was then blended with basal diet to give the 3000 ppm high-dose diet. This was diluted to give the 1000, 300 and 100 ppm diets. Preparation of the test diets was undertaken once every 4 weeks, and they were then stored at 4 °C before use. Control animals were fed basal diet without supplement. Animals were given fresh diet every week and fed ad lib.

2.4. Experimental design

Young adult and aged rats received diets containing oxolinic acid at 0 (control), 300, 1000 or 3000 ppm for 4 weeks, and the basal diet or the highest dose for 13, 26 or 52 weeks (52 weeks; only aged rats). Body weights were measured every week throughout the study, together with food consumption for the first 26 weeks. After 4 weeks of treatment, eight animals in each treated and control group were euthanized by decapitation and necropsied. After 13, 26 or 52 (only aged rats) weeks of treatments, six to 10 animals per group were similarly examined. At autopsy, the testes, epididymides, prostate (ventral lobule), seminal vesicles, brain and pituitary of each animal were removed and weighed.

2.5. Histopathological examination

The left testis and epididymides were fixed in Bouin's fixation for 24 h and the other organs in 10% formaldehyde solution. They were all routinely processed and stained with H&E for histopathological examination. Cross sections of the testis were stained with PAS reaction for morphometric analysis of spermatogenesis.

2.6. Proliferative lesions of Leydig cells

Aggregates of over 20 Leydig cells with abundant cytoplasm similar to focal hyperplasia but smaller in size than are 0.25 of a tubule were distinguished from normal Leydig cells and defined as foci. Lesions from 0.25 to three tubules in size and greater were respectively considered as focal hyperplasias and adenomas, in accordance with the WHO/STP classification of IARC (Mohr, 1997).

2.7. Morphometric analysis of spermatogenesis

To analyze the effects of exposure to oxolinic acid on spermatogenesis, selected cross sections from each right testis were examined in the control and 3000 ppm groups. The standard procedure is that if any effects are observed at the highest dose, the analysis is also performed

for the remaining groups. Morphometric analysis was performed with spermatogenesis stage waves divided into five stages, I–III, IV–VI, VII–VIII, IX–XI and XII–XIV, modifying the criteria reported by Clermont (1972) and Takahashi and Matsui (1993). Briefly, five round-shaped seminiferous tubules were randomly selected for each animal and the numbers of seminiferous epithelial and Sertoli cells in each stage were counted. Average numbers per seminiferous tubule were calculated as individual data at each stage. For Leydig cells, the numbers of cells in 10 clusters observed in the testicular interstitium were counted. Animals found dead or killed when moribund were also examined histopathologically.

2.8. Hormone assay

To analyze the effects of pituitary and testicular hormones, blood samples were collected from all animals on decapitation, centrifuged at 3000 rpm for 10 min and the serum stored at −70 °C until use. Serum LH was measured with NIADDK-rat-FSH and -LH radioimmunoassay (RIA) kits (NIAMDD, NIH, Bethesda, MD, USA). Serum testosterone levels were measured using a RIA technique.

2.9. Statistical analysis

Data for body and organ weights, food consumption, morphometrical analysis of spermatogenesis and Leydig cells, hormone levels and PCNA values were evaluated by analysis of variance followed by the Dunnett's t-test (4 weeks treatment) or the Student's t-test (13, 26 or 52 weeks treatment). The incidences of pathological findings and the numbers of foci and focal hyperplasias of Leydig cells were analyzed using the Fisher's exact test or the chi-square test. Probability values of $P<0.05$ were considered to be statistically significant.

3. Results

Data for body weights and food consumption in young adult and aged rats are shown in Figs. 1 and 2, respectively. Statistically significant depression of body weight was observed in the 3000 ppm group of young adult and aged rats and especially the latter throughout the study, although the food intake was increased at most time points. In the other treated groups, body weights and food consumption were comparable with those of the control groups. No other clinical signs related to the treatment were evident in any of the treated animals throughout the treatment period.

Macroscopically, no dose-dependent changes were found. In some examined points, weights of the testis, prostate or seminal vesicles were varied due to atrophic changes in aged rats including the control group. However, both absolute and relative organ weights for the testis, epididymides, prostate and seminal vesicles in young adult and aged treated groups were not significantly different from those in the relevant controls at all examined time points, as shown in Table 1.

Histopathological findings for the testes are shown in Table 2. In young adult rats, only foci of Leydig cells

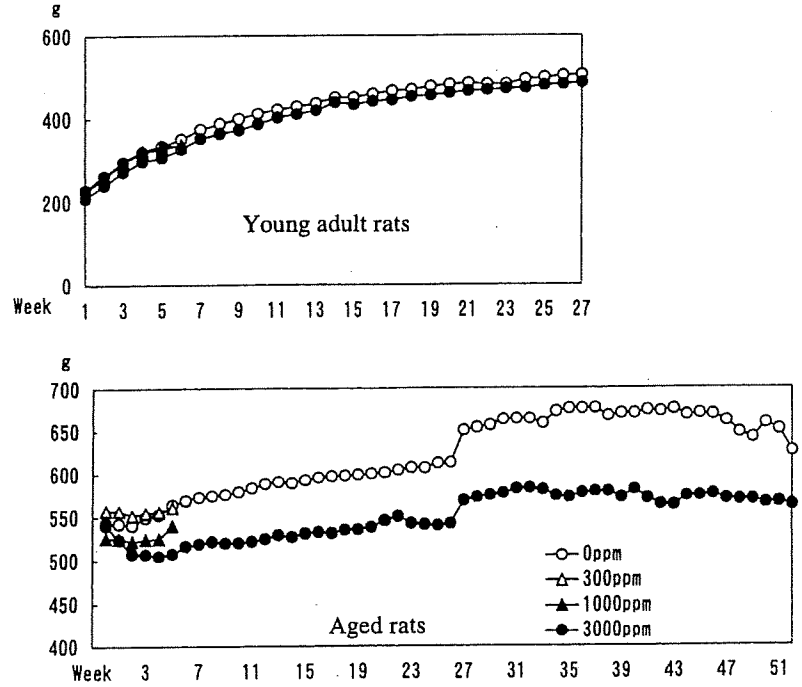

Fig. 1. Growth curves of young adult and aged rats.

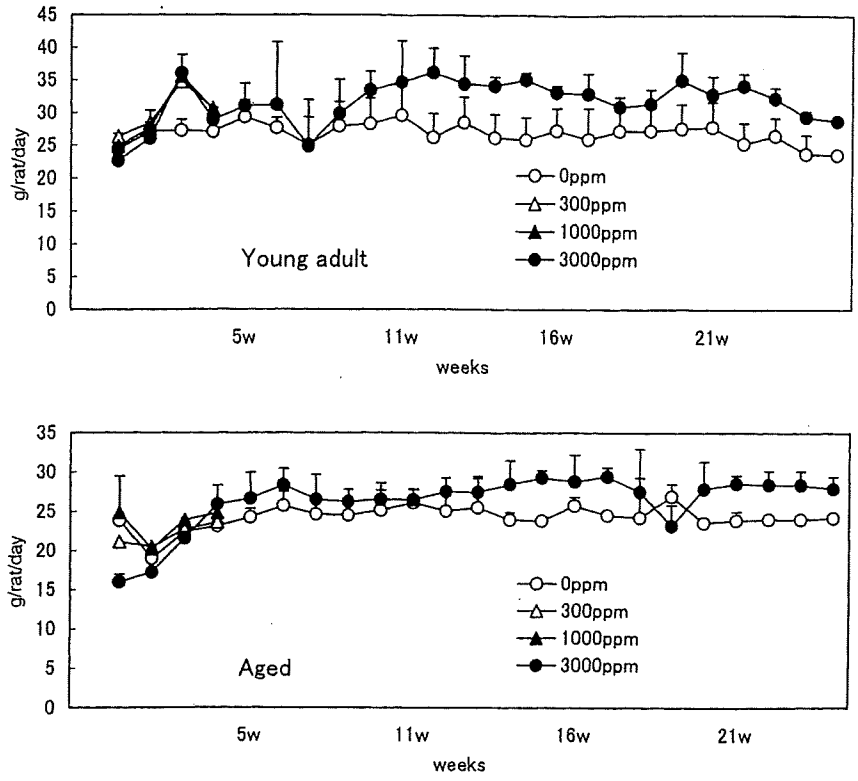

Fig. 2. Food consumption of young adult and aged rats.

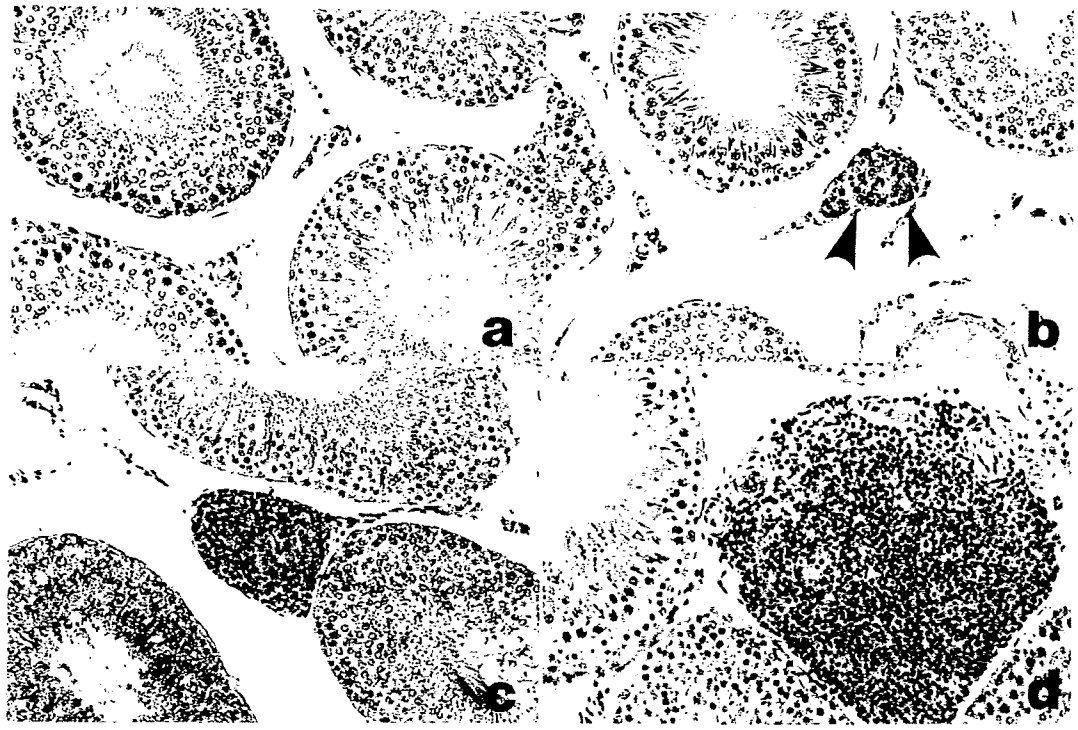

Plate 1. (a) Normal testis interstitum; (b) focus of Leydig cells; aggregates of over 20 cells with vacuolated round cytoplasm and enlarged nuclei (arrowheads) in aged rats of the control group at 26 weeks; (c) focal hyperplasia more than a 0.25 tubule but not exceeding 3 tubules in size; (d) a Leydig cell tumor (adenoma) in aged rats of the 3000 ppm group at 52 weeks. (a–d), hematoxylin-eosin staining. ×33.

Table 1
Sequential changes in organ weights

Examined point/group (ppm)		Number of rats	Body weights (g)	Testes (mg)		Epididymides (mg)		Prostate (mg)		Seminal vesicles (mg)	
				Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
Young adult											
4 w	0	8	341±9.1	3159±171.5		954±76.0		729±159.3		1027±196.3	
				(92.79±5.23)[a]		(28.00±1.96)		(21.44±4.82)		(30.24±6.14)	
	300	8	341±17.3	3191±135.7		1005±76.5		738±87.7		965±169.4	
				(93.78±5.01)		(29.56±2.73)		(21.70±2.75)		(28.54±5.96)	
	1000	8	339±16.1	3250±143.9		933±93.9		684±85.6		886±180.5	
				(96.02±4.18)		(27.50±1.98)		(20.19±2.41)		(26.30±5.75)	
	3000	8	314±12.5[b]	3034±101.3		827±35.0		767±67.2		921±114.4	
				(96.79±3.37)		(26.39±1.20)		(24.42±1.38)		(29.32±3.06)	
13 w	0	6	440±21.3	3364±129.5		1478±165.1		1135±143.8		1613±122.4	
				(76.65±3.91)		(33.65±3.56)		(25.78±2.65)		(36.75±3.16)	
	3000	6	417±16.0	3455±111.7		1364±58		1160±117.3		1756±194.3	
				(83.00±1.97)		(32.82±2.14)		(27.82±2.29)		(42.28±5.39)	
26 w	0	10	506±27.3	3347±83.3		1327±68.9		1397±324.6		1644±283.5	
				(52.79±26.61)		(26.32±2.24)		(27.51±5.70)		(32.41±5.11)	
	3000	10	488±36.8	3430±113.1		1288±86.4		1234±133.5		1498±215.4	
				(62.94±21.87)		(26.58±2.85)		(25.41±2.96)		(30.96±5.35)	
Aged animals											
4 w	0	8	520±20.1	3294±101		1412±176.5		2655±1071.4		1800±134.1	
				(63.42±2.75)		(27.22±3.74)		(51.07±20.51)		(34.64±2.67)	
	300	8	554±36.0	3417±136.7		1355±65.5		1915±970.1		1745±441.4	
				(61.97±4.84)		(24.19±1.94)		(34.27±16.00)		(24.07±15.63)	
	1000	8	527±29.8	3285±160.1		1365±138.9		1473±345.9		1750±277.1	
				(62.56±4.68)		(25.91±2.02)		(27.85±5.79)		(33.39±5.99)	
	3000	8	485±20.6*	3330±217.3		1431±230.2		1471±239.0		1610±59.9	
				(68.80±5.72)		(29.57±5.24)		(30.28±4.52)		(33.19±5.38)	
13 w	0	6	532±50.1	3260±259.1		1370±69.0		1201±150.4		2083±129.5	
				(62.03±9.19)		(22.09±10.04)		(22.57±1.92)		(39.63±5.77)	
	3000	6	517±20.7	5902±5697.2		1383±62.2		1338±265.2		1913±136.1	
				(114.84±111.8)		(26.81±1.66)		(25.83±4.63)		(37.00±1.83)	
26 w	0	10	587±55.9	3451±121.8		1455±124.4		1433±263.4		1988±394.3	
				(59.36±6.46)		(24.95±2.78)		(24.55±4.42)		(34.44±9.01)	
	3000	9	524±36.5*	3260±213.3		1551±399.4		1353±228.5		1933±128.2	
				(62.29±2.96)		(29.61±7.41)		(25.96±4.86)		(37.05±3.50)	
52 w	0	7	628±97.0	3283±648.9		1451±219.5		634±132.8		1669±491.3	
				(51.97±10.81)		(23.27±2.79)		(10.31±2.62)		(16.03±11.53)	
	3000	8	551±72.0	3467±257.8		1403±174.0		702±198.9		1368±704.0	
				(63.45±5.18)		(26.31±2.96)		(13.46±4.49)		(25.77±15.99)	

[a] Relative data calculated by absolute weight (mg)/body weight (g)×10.
[b] Significantly different from value of the 0 ppm group ($P<0.05$).

(Plate 1) were observed at 26 experimental weeks, the incidences being essentially the same in both treated and control groups. In aged rats, foci and focal hyperplasias were observed in both 3000 ppm and control animals at all time points, and the incidences in the 3000 ppm group appeared to increase at 26 weeks and significantly at 52 weeks. In young adult rats, tubular degeneration and/or atrophy were found at 26 weeks but with no difference between control cases. Similarly, these changes in seminiferous tubules were sporadically observed in aged rats at all time points, but again no treatment-dependent increase was observed. Histopathologically, the distribution and degree of these changes in the testes were not correlated with those for the proliferative lesions of Leydig cells.

Results of morphometric stage analysis of spermatogenesis and the numbers of Leydig cells in young adult and aged rats treated with 0 and 3000 ppm oxolinic acid are shown in Figs. 3 and 4, respectively. Significant differences were observed at some time points but the spermatogenesis was consistently maintained, although gradually diminishing with age, especially after 52 weeks in the aged animals (24 months of age). The testes in aged rats often showed severe tubular atrophy or Sertoli's-only syndrome, but these changes were not increased in the 3000 ppm group and numbers of Leydig cells were comparable between treated and relevant control groups in both young adult and aged rats throughout. Additional morphometric stage analysis of spermatogenesis in the 300 and 1000 ppm groups was therefore not performed.

Table 2
Sequential histopathological changes in testis

Young adult: Dose (ppm):	4 W				13 W		26 W			
	0	300	1000	3000	0	3000	0	3000		
No. of animals	8	8	8	8	6	6	10	10		
Leydig cell:										
Focus	0	0	0	0	0	0	2	1		
Focal hyperplasia	0	0	0	0	0	0	0	0		
Seminiferous tubules:										
Degeneration		0	0	0	0	0	1	0		
Atrophy	0	0	0	0	0	0	2	2		
Aged	4 W				13 W		26 W		52 W	
Dose (ppm)	0	300	1000	3000	0	3000	0	3000	0	3000
No. of animals	8	8	8	8	6	6	10	9	7	8
Leydig cell:										
Focus	3	1	1	4	4	6	4	9*	3	8*
Focal hyperplasia		1	0	0	1	2	0	1	1	5*
Adenoma	0	0	0	0	0	0	0	0	1	1
Seminiferous tubules:										
Degeneration		3	1	1	4	0	1	0	0	1
Atrophy	0	2	0	1	1	1	3	3	2	2

Significant differences at $P<0.05$.

Lesion of Leydig cells, focus, aggregates of over 20 Leydig cells with abundant cytoplasm, Hyperplasia, aggregates of Leydig cells from 1/4 to 3 tubules in size.

Adenoma, aggregates of Leydig cells greater than 3 tubules in size.

Tubules, degeneration, scattered degenerative changes partly observed in one tubule.

Atrophy, Sertoli only syndrome.

As to histopathological lesions observed in other genital organs, spermatogranulomas of the epididymis and focal chronic prostatitis were evident in all groups, including the controls. Pituitary hyperplasias and adenomas, chronic progressive nephropathy, myocardial fibrosis and other age-related lesions in many organs were observed at 52 weeks, but rarely appeared to be influenced by treatment.

Data for labeling indices (LIs) for cell proliferative activity in Leydig cells, assessed in PNCA positively, are shown in Table 3. Although values were significantly increased at 4 weeks in the 1000 and 3000 ppm groups of young adult rats, the change was not observed in older animals.

Changes in serum hormone levels of LH and testosterone are shown in Figs. 5 and 6. The LH levels in the 3000 ppm groups were higher than in the controls at 13 and 26 weeks in young adult rats, and at 4, 13 and 52 weeks in aged rats. The levels were comparable to the control levels in the 300 and 1000 ppm groups of both young adult and aged rats at 4 weeks. Serum testosterone levels were increased at 4 weeks (in young adult and aged rats) and at 13 weeks (in young adult rats). Thereafter, the levels were not different from those of the controls in either group.

4. Discussion

In the present study, while an increase in foci and/or focal hyperplasia of Leydig cells was noted in aged animals given oxolinic acid for 52 weeks, this was not related to changes in seminiferous tubules or spermatogenesis.

Prolonged treatment of oxolinic acid was earlier found to induce Leydig cell tumors in rats, and tubular atrophy of the testis was also increased significantly in the highest treated group at the termination of the carcinogenicity study (Yamada et al., 1994a). The mechanisms, as investigated by Yamada et al., appeared to involve an increase in serum LH, which was not influenced by daily administration of L-dopa or blocked by the injection of the dopamine antagonist, haloperidol, indicating no stimulation of LHRH release (Yamada et al., 1994b, 1995a,b). In a microdialysis study, Yamada et al. also found that extracellular concentration of dopamine in the preoptic area of the hypothalamus was elevated in rats given the compound (Yamada et al., 1995b). They concluded that the increase in serum LH level induced by oxolinic acid was mediated by facilitation of the dopamigenic system in the hypothalamic–pituitary axis rather than androgenic activity. However, direct effects of oxolinic acid on the seminiferous

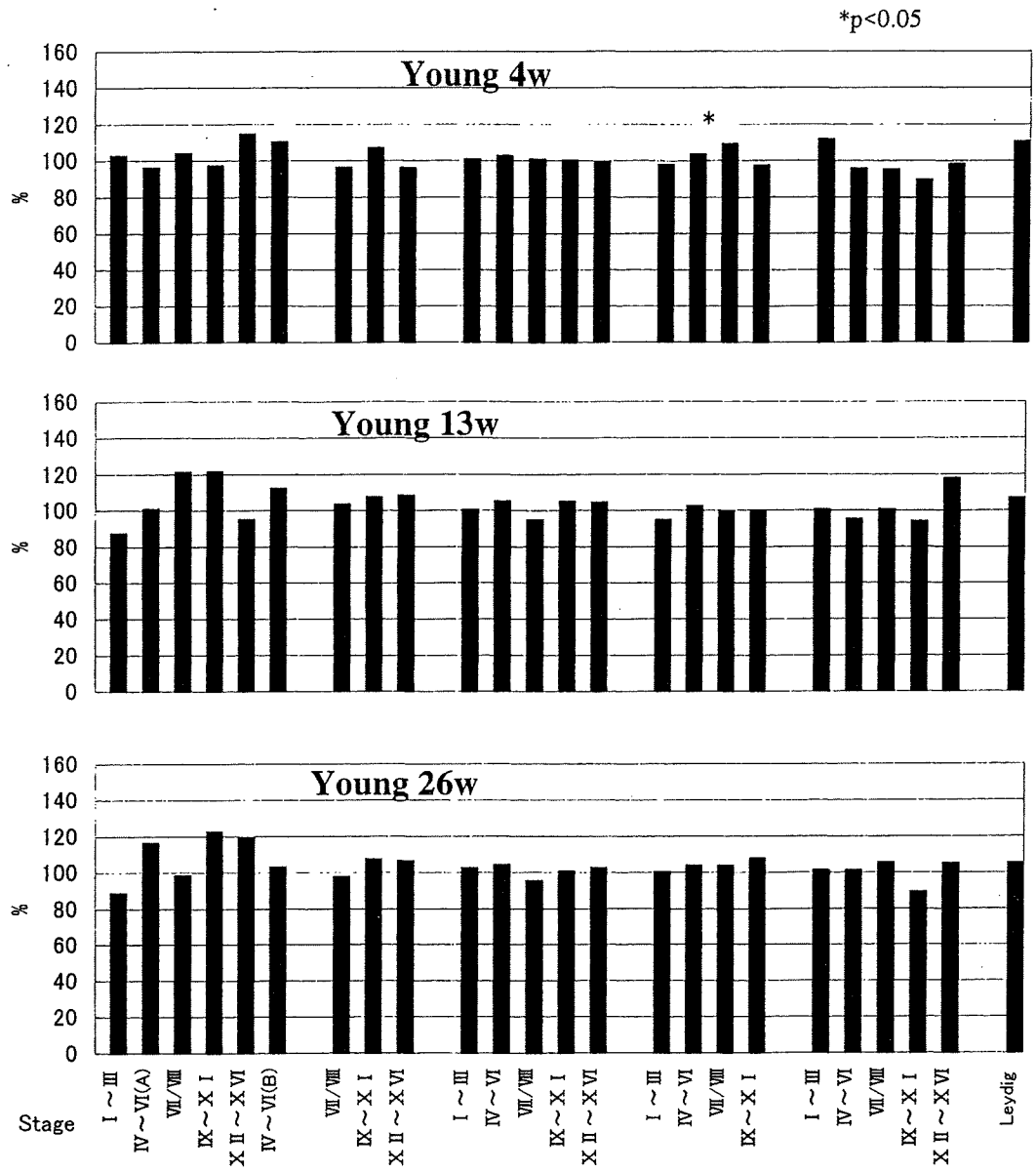

Fig. 3. Morphometrical stage analysis of spermatogenesis in young adults. Early cyte: preleptotene, leptotene or zygotene spermatocytes. Late cyte: pachytene or diplotene spermatocytes. Round tid: round spermatotid. Leydig: Leydig cells.

epithelium or a relationship between tubular atrophy and an increase of Leydig cell tumors were not precluded.

Our present results suggest that continuous administration of 3000 ppm oxolinic acid induces hyperplastic lesions of Leydig cells, but the carcinogenicity activity is very weak, and aged animals have higher sensitivity. Our data for PCNA labeling index in Leydig cells might support the indication of only a weak potential of oxolinic acid to induce Leydig cell tumors. Chatani et al. (1996) described increased LH levels to be essential for development and maintenance of naturally occurring Leydig cell tumors, one of the most common tumors in aged male rats (Boorman et al., 1990; Chatani et al., 1996). On the contrary, several compounds are known to induce the tumors by directly or indirectly mediated LH elevation (Roberts et al., 1989; Fort et al., 1995; Murakami et al., 1995; Hamada and Futamura, 1998). Yamada et al. found prolonged elevation of LH levels due to oxolinic acid tightly linked with induction of Leydig cell tumors (Yamada et al., 1994b), in line with our findings. While we noted increases of testosterone levels at early time points, no change was evident in older animals. The two hormones might be independently regulated for development of Leydig cell tumors.

With regard to discrepancies between our results and the previous report (Yamada et al., 1994a), first we noted that the incidences of proliferative lesions of Leydig cells in the 3000 ppm group were here lower,

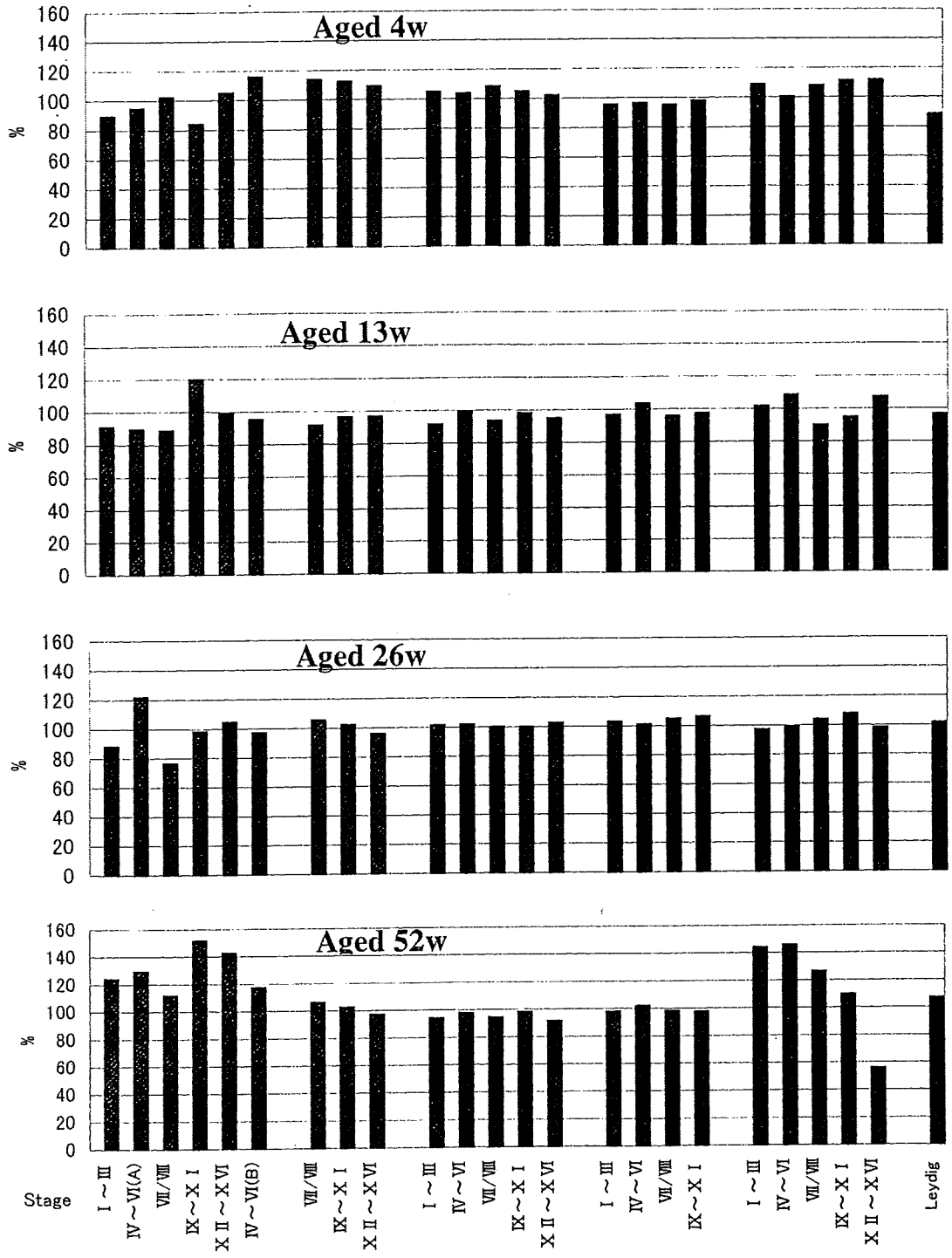

Fig. 4. Morphometrical stage analysis of spermatogenesis in aged rats. Early cyte: preleptotene, leptotene or zygotene spermatocytes. Late cyte: pachytene or diplotene spermatocytes. Round tid: round spermatid. Leydig: Leydig cells.

especially at 52 weeks in aged rats (104 weeks of age). For one reason, the treatment period in our study, 52 weeks, might be too short to induce adequate numbers of Leydig cell tumors compared with the previous carcinogenicity study for 104 weeks (Yamada et al., 1994a). Secondly, the LH levels in the 1000 ppm groups of young adult and aged rats were comparable to those in our relevant controls at 4 weeks, although this dose caused increase of LH levels in previous investigations (Yamada et al., 1994a,b).

The morphometric analysis method performed by assessment of seminiferous epithelium at each stage of spermatogenesis was established by Clermont et al. (Clermont, 1972; Russell, 1990), and is now widely

Table 3
Sequential changes in PCNA-positive cells (%)

Young adult:	4 W				13 W		26 W	
Dose (ppm):	0	300	1000	3000	0	3000	0	3000
No. of animals	8	8	8	8	6	6	10	10
% of PCNA cells								
Mean	2.71	2.5	3.04	3.56	2.4	2.49	2.1	1.85
S.D.	0.584	0.767	0.511	0.538	0.486	0.606	0.453	0.686

Aged animals:	4 W				13 W		26 W	
Dose (ppm)	0	300	1000	3000	0	3000	0	3000
No. of animals	8	8	8	8	6	6	10	9
% of PCNA-positive cells								
Mean	3.36	3.12	4.27	3.17	2.4	2.33	1.23	1.05
S.D.	0.962	1.426	1.111	1.407	0.551	0.558	0.773	0.328

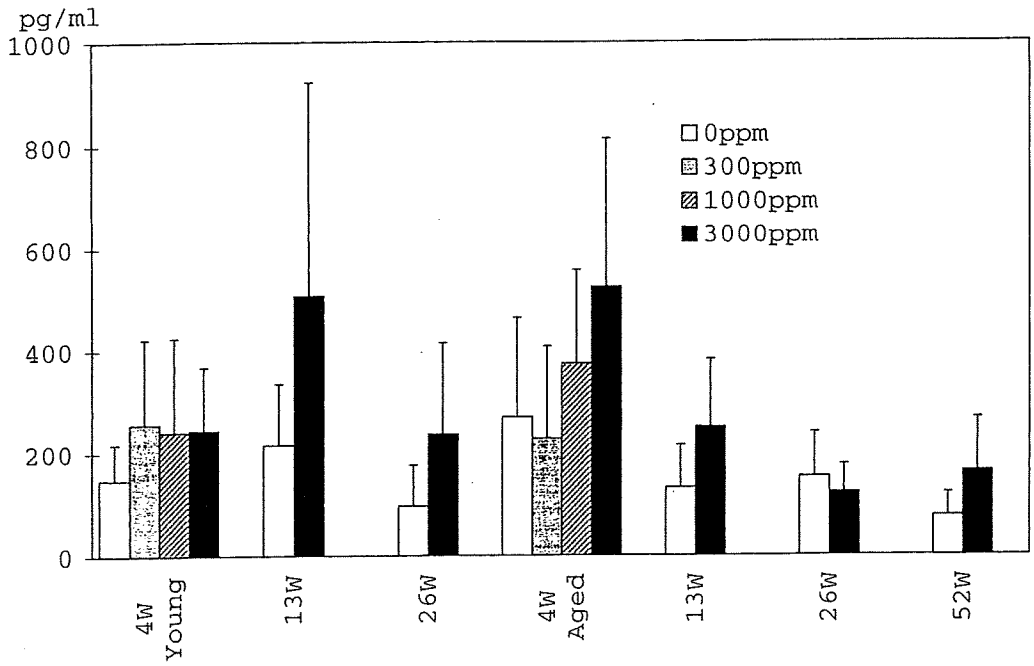

Fig. 5. Serum luteinizing hormone levels in young-adult and aged rats.

considered to be the most sensitive and useful tool to detect testicular damage (Russell, 1990; Takahashi and Matsui, 1993; Takayama et al., 1995). In the present study, significant differences observed in the morphometric analysis for spermatogenesis were not related the treatment period in either young adult or aged rats, indicating that oxolinic acid was without influence, in agreement with the data for tubular degeneration and atrophy. Therefore, the results described above clearly demonstrate that oxolinic acid does not affect directly the seminiferous epithelium and spermatogenesis in either young adult or aged rats.

As one result of indirect toxicity of chemicals, it is known that a decline in testosterone levels caused by modulation of gonadotropin results in abnormal spermatogenesis mediated via Leydig-Sertoli cells (Dickerman et al., 1974; Kohge et al., 2000). However, the present study could not detect any findings that oxolinic acid contributed altered seminiferous tubules via modulation of hormonal environment. In addition, no

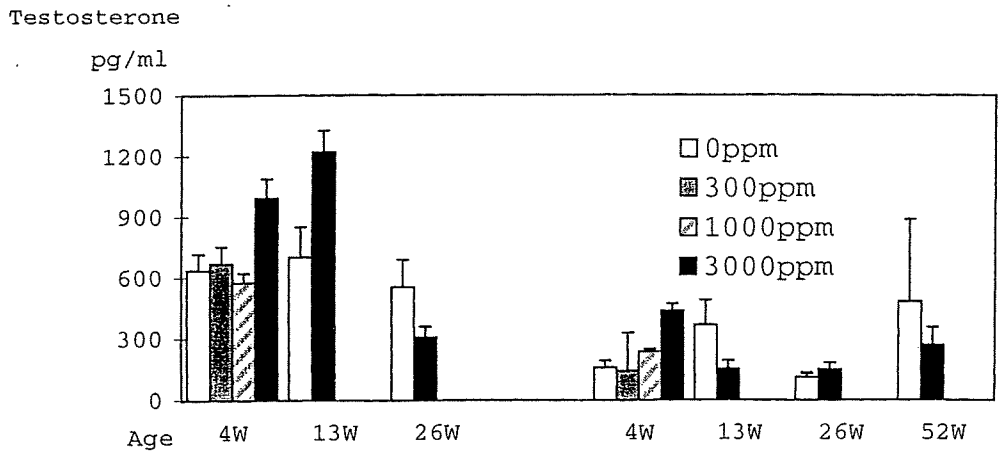

Fig. 6. Serum testosterone levels in young adult and aged rats.

positive relationship between the distribution or degree of tubular damage and the development of proliferative lesions of Leydig cells could be detected.

While continuous body weight depression related to the treatment was observed in rats receiving 3000 ppm in groups of rats in both age groups, this clearly did not exert a major effects on spermatogenesis and representative organs did not show any decrease in their weights. In conclusion, the present study provides evidence that prolonged oxolinic acid treatment has no direct or indirect influences on spermatogenesis and seminiferous tubules in either young adult or aged rats. In addition, it confirmed that oxolinic acid induces proliferative lesions of Leydig cells via increase of serum LH levels, being more sensitive in aged rats in this respect.

Acknowledgements

This study was supported by a Grant-in-Aid from the Ministry of Health Labor and Welfare of Japan. We gratefully express an appreciation to Mrs Hiromi Ichihara for expert technical assistance for the histopathological examination.

References

Boorman, G.A., Chapin, R.E.K., 1990. Testis and epididymis. In: Boorman, G.A., Eustis, S.L., Elwell, M.R., Montgomery Jr., C.A., MacKenzie, W.F. (Eds.), Pathology of the Fischer Rat. Academic Press, San Diego, pp. 405–418.

Chatani, F., Nonoyama, T., Sudo, K., Miyajima, H., Takeyama, M., Takatsuka, D., Mori, H., Matsumoto, K., 1990. Stimulatory effect of lutenizing hormone on the development and maintenance of 5-reduced steroid-producing testicular interstitial cell tumors in Fischer 344 rats. Anticancer Research 10, 337–342.

Chatani, F., Suzuki, S., Hamajima, T., Orita, S., 1996. Age-related changes in serum LH and testosterone in 5–110 week-old male F344, Wistar and Sprague-Dawley rats. Journal of Takeda Research Laboratory 55, 188–200.

Clermont, Y., 1972. Kinetics of spermatogenesis in mammals: seminiferous epithelium cycle and spermatogonial renewal. Physiological Reviews 52, 198–236.

Cozzarelli, N.R., 1980. DNA gyrase and the supercoiling of DNA. Science 207, 953–960.

Dickerman, S., Kledzik, G., Gelato, M., Chen, H.J., Meites, J., 1974. Effects of haloperidol on serum and pituitary prolactin, LH and FSH, and hypothalamic PIF and LRF. Neuroendocrinology 15, 10–20.

Fort, F.L., Miyajima, H., Ando, T., Suzuki, T., Yamamoto, M., Hamashima, T., Sato, S., Kitazaki, T., Mahony, M.C., Hodgen, G.D., 1995. Mechanism for species-specific induction of Leydig cell tumors in rats by lansoprazole. Fundamental and Applied Toxicology 26, 191–202.

Gleckman, R., Alvarez, S., Joubert, D.W., Matthew, S.J., 1979. Drug therapy reviews: oxolinic acid. American Journal of Hospital and Pharmacology 36, 1077–1079.

Hamada, Y., Futamura, Y., 1998. Induction of Leydig cell tumors by lacidipine via up-regulation of the LHRH receptor of Leydig cells in rats. Journal of Toxicological Sciences 123, 35–52.

Hikichi, Y., Noda, C., Shimizu, K., 1989. Oxolinic acid. Japan Pesticide Information 55, 21–23.

Hosokawa, S., Murakami, M., Ineyama, M., Yamada, T., Koyama, Y., Okuno, Y., Yoshitake, A., Yamada, H., Miyamoto, J., 1993. Effects of procymidone on reproductive organs and serum gonadotropins in male rats. Journal of Toxicological Sciences 18, 111–124.

Kohge, S., Hagi, S., Utsumi, H., Takegawa, K., Takagi, H., Nagaoka, T., Takeuchi, M., Hanada, S., 2000. Collaborative work to evaluate toxicity on male reproductive organs by repeated dose studies in rats 6)2- and 4-weeks administration study of haloperidol. Journal of Toxicological Sciences 25 (Special Issue), 71–77.

Mohr U, (Ed in chief), 1997. Testis. In: International Classification of Rodent Tumors. Part 1. The Rat. 8. Male Genital System. IARC Scientific Publications No. 122. International Agency for Research on Cancer, Lyon, pp. 1–22.

Murakami, M., Hosokawa, S., Yamada, T., Harakawa, M, Ito, M., Koyama, K., Kimura, J., Yoshitake, A., Yamada, H., 1995. Species-specific mechanism in rat Leydig cell tumorigenesis by procymidone. Toxicology and Applied Pharmacology 131, 244–252.

Osheroff, N., Shelton, E.R., Brutlag, D.L., 1983. DNA topoisomerase from Drosophila melanogaster. Journal of Biological Chemistry 258, 9536–9543.

Roberts, S.A., Nett, T.M., Hartman, H.A., Adams, T.E., Stoll, R.E., 1989. SDZ 200–100 induces Leydig cell tumors by increasing gonadotropins in rats. Journal of the American College of Toxicology 8, 487–505.

Russell, L.D., 1990. The Sertoli cell. In: Russell, L.D., Ettlin, R.A., Hikim, A.P.S., Clegg, E. (Eds.), D (Eds.), Histological and Histopathological Evaluation of the Testes. Cache River Press, FL, pp. 29–36.

Takahashi, M., Matsui, H., 1993. Mechanisms of testicular toxicity. Journal of Toxicologic Pathology 6, 161–174.

Takayama, S., Akaike, M., Kawashima, K., Takahashi, M., Kurokawa, Y., 1995. A collaborative study in Japan on optimal treatment period and parameters for detection of male fertility disorders induced by drugs in rats. Journal of the American College of Toxicology 14, 266–292.

Yamada, T., Maita, K., Makamura, J., Murakami, M., Okuno, Y., Hosokawa, S., Matsuo, M., Yamada, H., 1994a. Carcinogenicity studies of oxolinic acid in rats and mice. Food and Chemical Toxicology 32, 397–408.

Yamada, T., Nakamura, J., Murakami, M., Okuno, Y., Hosokawa, S., Matsuo, M., Yamada, H., 1994b. The correlation of serum lutenizing levels with the induction of Leydig cell tumors in rats by oxolinic acid. Toxicology and Applied Pharmacology 129, 146–154.

Yamada, T., Nakayama, J., Murakami, M., Okuno, K., Hosokawa, S., Matsuo, M., Yamada, H., 1995a. Effect of chronic L-DOPA administration on serum luteinizing hormone levels in male rats. Toxicology 97, 173–182.

Yamada, T., Nakamura, J., Okuno, Y., Hosokawa, S., Matsuo, M., Yamada, H., Ohta, M., 1995b. A possible mechanism for the increase in serum luteinizing hormone levels in male rats by oxolinic acid. Toxicology and Applied Pharmacology 134, 35–42.

Journal of Andrology, Vol. 23, No. 6, November/December 2002
Copyright © American Society of Andrology

Inhibin B Regulating Follicle-Stimulating Hormone Secretion During Testicular Recrudescence in the Male Golden Hamster

WANZHU JIN,*† CHANDANA B. HERATH,† MIDORI YOSHIDA,‡ KOJI Y. ARAI,§ ERINA SAITA,*† SHI ZHANQUAN,*† LONGQUAN REN,*† GEN WATANABE,*† NIGEL P. GROOME,‖ AND KAZUYOSHI TAYA*†

*From the *Department of Basic Veterinary Sciences, The United Graduate School of Veterinary Sciences, Gifu University, Gifu, Japan; the †Laboratory of Veterinary Physiology and the §Department of Tissue Physiology, Tokyo University of Agriculture and Technology, Tokyo, Japan; the ‡Department of Pathology, Sasaki Institute, Tokyo, Japan; and the ‖School of Biological and Molecular Science, Oxford Brookes University, Oxford, United Kingdom.*

ABSTRACT: In the present study, to clarify whether inhibin affects follicle-stimulating hormone (FSH) secretion in the recrudescence of the male golden hamster, we used a recently developed specific enzyme-linked immunosorbent assay (ELISA) in order to measure 2 forms of inhibin molecules: inhibin B and inhibin pro-αC. In addition, we used the radioimmunoassay (RIA) to measure immunoreactive (ir-)inhibin, FSH, luteinizing hormone (LH), and testosterone. And finally, we used the proliferating cell nuclear antigen (PCNA) and computer-assisted sperm motion analysis (CASA) methods to ascertain how well spermatogenesis and sperm motility recover from the photoinhibition caused by exposure to a short-day (SD; 10-hour light:14-hour dark) photoperiod. Animals were exposed to SD for 15 weeks, and then their testes were checked carefully and found to be completely regressed. Thereafter, those animals were transported to a long-day (LD; 14-hour light:10-hour dark) photoperiod. Sampling was carried out at weeks 0 (exposed SD 15 weeks), 1, 2, 4, 6, 8, and 10. Plasma FSH rapidly increased and reached peak levels 2 weeks after transferral to the LD photoperiod and then declined to normal LD levels at week 6. Circulating ir-inhibin, inhibin B, and inhibin pro-αC rose to normal LD levels by week 4. A highly significant inverse correlation was observed between plasma FSH and inhibin B but not between FSH and either ir-inhibin or inhibin pro-αC. Plasma testosterone recovered to normal LD levels within 1 week. Sperm motility parameters were low until week 2 and recovered to normal LD levels from weeks 4 to 10. PCNA-labeled cells were confined to the spermatogenic cells of the seminiferous tubules, though Leydig and Sertoli cell nuclei were never stained for PCNA during the period studied. The number of pachytene spermatocytes and the diameter of seminiferous tubules increased in a time-dependent manner after transferral from SD to LD. In conclusion, these results suggest that 1) secretion of inhibin B may be stimulated by an early rise in FSH; 2) inhibin B suppresses FSH secretion from weeks 2 to 10, after transferral to the LD photoperiod; and 3) testes recrudescence is based on the increase in the number of sperm cells instead of the increase in the number of Sertoli and Leydig cells of the male golden hamster.

Key words: Photoperiod, proliferating cell nuclear antigen, sperm motion.

J Androl 2002;23:845–853

The golden hamster, unlike most commonly used laboratory rodents, has been an invaluable model for studies of the seasonal pattern of reproduction. In male hamsters, exposure to a short photoperiod has been found to induce morphological and functional regression of the reproductive system (Berndtson and Desjardins, 1974; Turek et al, 1975; Goldman et al, 1981). Continuous exposure to inhibitory photoperiods causes hamsters to become insensitive to the light regimen, probably because of the interruption of nocturnal pineal melatonin synthesis (Lerchl and Nieschlag, 1992). Conversely, it has been demonstrated that when hamsters are transferred from a short-day (SD) photoperiod to a long-day (LD) photoperiod, plasma follicle-stimulating hormone (FSH), luteinizing hormone (LH), prolactin, testosterone, and immunoreactive (ir)-inhibin return to normal levels, inducing the recovery and reestablishment of testicular functions (Berndtson and Desjardins, 1974; Turek et al, 1975; Matt and Stetson, 1980; Goldman et al, 1981; Simpson et al, 1982; Milette et al, 1988; Kirby et al, 1993). However, it is evident that the rise in plasma FSH precedes any change in plasma levels of LH and testosterone during photoperiod-induced spontaneous testicular recrudescence in male golden hamsters (Turek et al, 1975; Milette et al, 1988; Kirby et al, 1993; Donham et al, 1994). It has been suggested that an early rise in FSH secretion is primarily dependent upon endogenous gonadotropin-releasing hormone (GnRH) release (Meredith et al, 1998).

It is well known that inhibin is a heterodimeric protein

Supported in part by a US-Japan cooperative research grant from the Japan Society for the Promotion of Science and a grant-in-aid (GAKUCHO SAIRYO KEIHI) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

Correspondence to: Dr Kazuyoshi Taya, Laboratory of Veterinary Physiology, Tokyo University of Agriculture and Technology, 3-5-8 Saiwai-cho, Fuchu, Tokyo 183-8509, Japan (e-mail: taya@cc.tuat.ac.jp).

Received for publication February 21, 2002; accepted for publication June 6, 2002.

consisting of an α subunit and one of 2 β subunits. Two related forms of inhibin, inhibin A (α/βA) and inhibin B (α/βB), are secreted into the circulation from the gonads and inhibit pituitary FSH secretion (Ling et al, 1985; Miyamoto et al, 1985; Rivier et al, 1985; Robertson et al, 1985; Vale et al, 1986). A recently developed specific immunoassay has enabled the measurement of plasma levels of dimeric inhibins and provided evidence that inhibin B is an important physiological form of inhibins in male golden hamsters (Jin et al, 2001b), as well as in other males: male Gottingen miniature pigs (Jin et al, 2001a), men (Illingworth et al, 1996), male monkeys (Foppiani et al, 1999; Ramaswamy et al, 2000), male chimpanzees (Kondo et al, 2000), and male rats (Sharpe et al, 1999). It has also been suggested that in men, inhibin B regulates FSH secretion (Anawalt et al, 1996; Illingworth et al, 1996; Nachtigall et al, 1996; Seminara et al, 1996). However, the existence of negative feedback of inhibin B on FSH secretion during photoperiod-induced testicular recrudescence in golden hamsters has been disputed and is yet to be elucidated. Kirby et al (1993) suggested that testicular ir-inhibin secretion may not be directly related to circulating FSH levels during recrudescence in the golden hamster.

The proliferating cell nuclear antigen (PCNA), a 36-kd acidic nuclear protein that has been very highly conserved in the process of evolution, is a cell proliferation marker currently drawing attention. PCNA is known to function as a cofactor for DNA polymerase δ, with a biological half-life of longer than 20 hours (Bravo and Macdonald-Bravo, 1987). PCNA is required for both DNA replication and DNA repair (Shivji et al, 1992; Xiong et al, 1992). It is synthesized primarily during the G1 phase of the cell cycle and reaches its maximum levels during the S phase (Hofstadter et al, 1995). Given adequate fixation and tissue processing, the results of PCNA immunohistochemistry directly reflect the proliferative status of the cells and the fact that PCNA-labeled nuclei are observed during the G1 to the S phase of the cell cycle (Morita et al, 1994). The localization of PCNA can be used to assess the proliferative status of renewing spermatogonia and to analyze the proliferative activity of the seminiferous epithelium of rodents and non-human primates (Schlatt and Weinbauer, 1994) and men (Garrido et al, 1992). Thus, PCNA expression levels in the cell nuclei are indicative of the proliferating activity of the renewing spermatogenic cells.

Therefore, in the present study, we tested the hypothesis that triggering the secretion of inhibins and the resulting increased levels of inhibins subsequently affect the FSH during the photoperiod-induced testicular recrudescence in the male golden hamster. Furthermore, renewing spermatogenic cell proliferating activity was evaluated by PCNA. and sperm motility characteristics were also monitored by a computer-assisted sperm analysis system (CASA) to ascertain how well the spermatogenesis recovers from the photoinhibition caused by exposure to an SD photoperiod.

Materials and Methods

Animals and Blood Samples

Adult male golden hamsters (*Mesocricetus auratus*) (3 months old) were used in the present study. The experimental design was based on a previous study by Donham et al (1994), who reported that the SD photoperiod-induced testicular regression was completed by 15 weeks. Thus, in the present study, experimental animals were kept under an SD photoperiod (10-hour light:14-hour dark cycles; lights on at 0900 hours) for 15 weeks. Upon complete regression of the testes at 15 weeks, the animals were transferred to an LD photoperiod (14-hour light:10-hour dark cycles; lights on at 0500 hours). The sampling was carried out at weeks 0, 1, 2, 4, 6, 8, and 10 (week 0 is the 15th week of the SD photoperiod). At each time point, 5 animals were killed by decapitation between 0900 and 1100 hours. Blood was collected, stored in ice, and centrifuged at 1700 × *g* for 15 minutes at 4°C. Plasma was separated and stored at −20°C until assayed for inhibins, LH, FSH, and testosterone. Control animals of the same age were housed in groups of 6 to 8 animals per cage in a room with controlled conditions of temperature, humidity, and lighting (14-hour light:10-hour dark cycles; lights on at 0500 hours). Food and water were available ad libitum. All experimental procedures involving animals were carried out in accordance with requirements established under the Guide for the Care and Use of Laboratory Animals by the Tokyo University of Agriculture and Technology. The experiment was repeated, and the data were found to be reproducible.

Radioimmunoassays for FSH, LH, Testosterone, and ir-Inhibin

Plasma concentrations of FSH and LH were measured using National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) radioimmunoassay (RIA) kits for rat FSH and LH, as described previously (Bast and Greenwald, 1974). The antisera used were anti-rat FSH (S-11) and LH (S-10). Results were expressed in terms of NIDDK rat FSH (RP-2) and LH (RP-2). The intra- and interassay coefficients of variation were 4.4% and 14.6% for FSH and 6.7% and 8.9% for LH, respectively.

Plasma concentrations of ir-inhibin were measured by a double-antibody RIA, as described previously (Hamada et al, 1989). The antiserum used was raised in rabbits against bovine inhibin (TNDH-1). Purified bovine 32-kd inhibin was used as the standard. The assay system does not distinguish dimeric inhibin from α subunit monomer. The intra- and interassay coefficients of variation were 8.8% and 14.4%, respectively.

Plasma concentrations of testosterone were determined by a double-antibody RIA system using ^{125}I-labeled radioligand, as described previously (Taya et al, 1985). Antiserum against testosterone was kindly supplied by Dr G. D. Niswender (Animal Reproduction and Biotechnology Laboratory, Colorado State

University, Fort Collins, Colo) (Gay and Kerlan, 1978). The intra- and interassay coefficients of variation were 6.3% and 7.2%, respectively.

Enzyme-Linked Immunosorbent Assay

Plasma concentrations of inhibin B and inhibin pro-αC were determined using enzyme-linked immunosorbent assay (ELISA) kits (Serotec Ltd, Oxford, United Kingdom). Inhibin A was not measured because we have previously reported that plasma inhibin A is not detectable in male golden hamsters (Jin et al, 2001b). We have validated and reported these inhibin dimer-specific assays for male golden hamsters (Jin et al, 2001b).

Tissue Processing

In each case, the right testis, including the epididymis, the seminal vesicle, and the coagulating gland complex, was weighed, and sperm from the right epididymis were used for sperm motion analyses. Testicular tissue samples were immediately fixed in freshly prepared 4% paraformaldehyde (Sigma Chemical Co, St Louis, Mo) in 0.05 M phosphate-buffered saline, pH 7.4, and embedded in paraffin. The paraffin-embedded testicular tissues were serially sectioned at 6-μm thickness and mounted onto poly-L-lysine (0.01% [wt/vol]) (Sigma) coated slide glasses (Dako Japan Co, Kyoto, Japan) for use in immunohistochemistry.

Immunohistochemistry for PCNA

After being deparaffinized with xylene, the tissue sections were subjected to antigen retrieval by autoclaving in 0.01 M sodium citrate buffer, pH 6.0, at 121°C for 15 minutes. Sections were then incubated in 6% H_2O_2 in methanol at room temperature for 1 hour, followed by 0.5% casein-Tris saline (0.05 M Tris-HCl with 0.15 M NaCl, pH 7.6; CTS) at 37°C for 1 hour, to quench nonspecific staining. Then, the tissue sections were incubated at 37°C for 16 to 18 hours with a monoclonal antibody raised against PCNA (Biomeda, Forster City, Calif) at a dilution of 1:200 in CTS. After incubation with the antibody, sections were treated with 0.25% (vol/vol) biotinylated goat anti-mouse secondary antibody (Elite ABC kit, Vector Laboratories, Burlingame, Calif) in CTS at 37°C for 1 hour. These sections were subsequently incubated with 2% (vol/vol) avidin-biotin complex (Elite ABC kit) in CTS at 37°C for 30 minutes. The reaction products were visualized by treatment with 0.025% (wt/vol) 3.3′-diaminobenzidine tetrachloride (DAB; Sigma) in 100 mM Tris-buffered saline containing 0.01% H_2O_2 for 1 to 30 minutes.

Computer-Assisted Sperm Mobility Analyses

The sperm motility parameters were obtained using the C. IMAGING CASA system. Sperm from the cauda epididymis were incubated at 37°C for 3 minutes in 0.01 M medium buffer, pH 7.2. The medium buffer was made up of 59.8 mg of HEPES (Dojindo, Kumamoto, Japan), 982 mg of medium 199 (Biocell, Carson, Calif), 500 mg of bovine serum albumin (Sigma), and 220 mg of $NaHCO_3$ (Wako, Osaka, Japan) dissolved in 100 mL deionized water. After the sperm were incubated in medium buffer at 37°C, an aliquot of this solution was diluted 10- to 20-fold, and 10 μL was placed into the microcell-HAC chamber, which has a depth of 50 μm (Conception Technologies, San Diego, Calif). Analyses of motility characteristics were performed on at least 200 cells for each sample. Sperm motion, as viewed on an Olympus microscope (4×, pseudodark-field optics) with a stage warmer (37°C) (MP-10DM; Kitazato Supply Co, Kitazato, Japan), was analyzed using the C. IMAGING system. The C. IMAGING system settings were as follows: frames analyzed, 15; framing rate, 30; maximum velocity, 1200 μm/s; threshold velocity, 45 μm/s; minimum linearity for ALH (amplitude of lateral head displacement), 3.5; pixel scale, 3.26 mm/pixel; maximum average number of cells/field, 30; and cell size range, 350 to 1600 pixels. The following characteristics were analyzed: percentage of motile spermatozoa, curvilinear velocity (total distance traveled divided by total time the cell was tracked), straight velocity (straight-line distance), mean ALH (deviation of the sperm head from the mean trajectory), max ALH (the maximum amplitude of lateral head displacement), linearity (ratio of the straight-line distance to the actual tracked distance), and percentage of circular cells.

Histological Analysis

The PCNA-labeled germ cells were counted under the microscope (Nikon, Tokyo, Japan). The diameters of 20 round tubules per animal were measured.

Statistics

A one-way analysis of variance was performed. Significance was determined by the Duncan multiple range test (Steel and Torrie, 1960). Correlation analysis between inhibins and FSH was performed using the Pearson method. All data are presented as the mean plus or minus the standard error of the mean. Differences were considered significant when P was less than .05.

Results

Changes in Weight of Reproductive Organs

Body weights (BWs) were not different between the treatment and control groups during the experimental period (Figure 1A). However, testes in the hamsters exposed to an SD photoperiod for 15 weeks displayed more than a 10-fold reduction ($P < .0001$) in weight. Relative testes (testes/BW) and epididymal (epididymal/BW) weights were significantly ($P < .001$) low in the treatment group compared with those in the control group until the fourth (testes) and sixth (epididymal) weeks. The weight of the testes and epididymis then began to recover and reached normal LD levels by 6 and 8 weeks, respectively (Figure 1B and D). However, the relative weight of the seminal vesicle (seminal vesicle plus coagulating gland complex) had not recovered to normal LD levels by 10 weeks (Figure 1C).

Changes in Plasma Concentrations of FSH, LH, ir-Inhibin, Inhibin B, Inhibin pro-αC, and Testosterone

The changes in plasma concentrations of reproductive hormones are shown in Figure 2. Rapid increases in con-

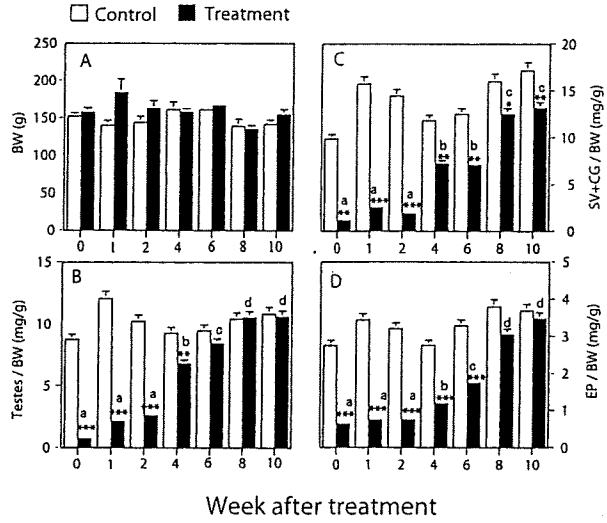

Figure 1. Changes in (A) body weights (BW), relative weights of (B) testes, (C) the seminal vesicle and coagulating gland complex (SV + CG), and (D) epididymis (EP). Each point represents the mean plus or minus the standard error of the mean from 5 animals. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .0001$, compared with the control group; different characters indicate a significant difference ($P < .05$) in each graph among the treatment group.

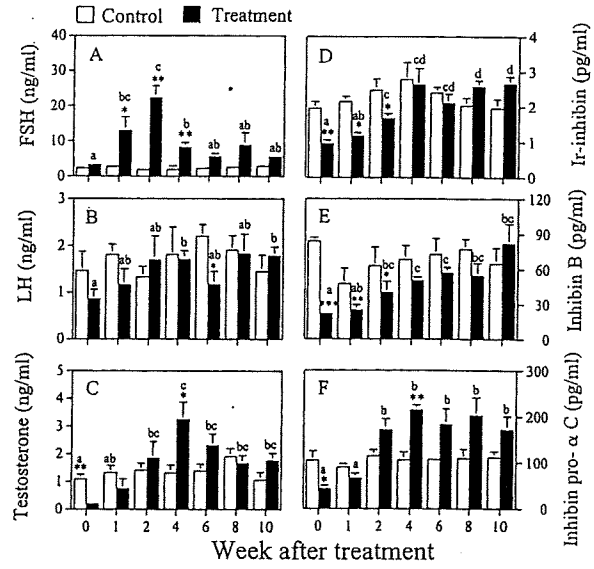

Figure 2. Changes in plasma concentrations of (A) follicle-stimulating hormone (FSH), (B) luteinizing hormone (LH), (C) testosterone, (D) immunoreactive (ir)-inhibin, (E) inhibin B, and (F) inhibin pro-αC during a 10-week period after the transfer from short-day (SD) to long-day (LD) photoperiods in male golden hamsters. Each point represents the mean plus or minus the standard error of the mean from 5 animals. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .0001$, compared with the control group; different characters indicate a significant difference ($P < .05$) in each graph among the treatment group.

centrations of plasma FSH occurred at 1 week after transferral from an SD to an LD photoperiod and reached peak levels (22 ng/mL) ($P < .01$) at week 2, followed by a rapid decline to normal LD levels (1.5–3 ng/mL) at week 6 (Figure 2A). In contrast, basal concentrations of plasma LH steadily increased after transferral from the SD to the LD photoperiod and were significantly high at weeks 4 and 10 compared with week 0, although these levels were not significantly different from those in the control group (Figure 2B). Plasma concentrations of testosterone were significantly ($P < .05$) low at week 0 in the treatment group compared with those in the control group. These concentrations then gradually increased and reached peak levels at week 4 before declining to normal LD levels (Figure 2C). Plasma concentrations of ir-inhibin (Figure 2D) and inhibin B (Figure 2E) began to increase at week 2 and reached normal LD levels at week 4. Plasma concentrations of inhibin pro-αC were significantly ($P < .05$) low at week 0 in the treatment group compared with those in the control group, but they then began to increase at week 1 and remained at higher levels than those in the control animals from weeks 2 to 10 (Figure 2F). Plasma FSH and inhibin B concentrations were inversely correlated ($r = -0.56$, $P < .01$) during the period from weeks 2 to 10 (Figure 3). However, neither ir-inhibin nor inhibin pro-αC was correlated with plasma FSH (data not shown).

Changes in Sperm Motility Parameters

No sperm were available in the cauda epididymis for motile parameter analysis until week 2, but 1 of the 5 animals had some sperm at week 2 (data not shown). The percentage of motile spermatozoa was abruptly increased at week 4 and reached normal LD levels at week 6 (Figure 4A). At week 10, the percentage of motile sperm was reduced slightly but significantly ($P < .05$) in the treatment group compared with that in the control group. The ALH mean (Figure 4B) and ALH max (Figure 4C) in the treatment group increased abruptly at week 4, although

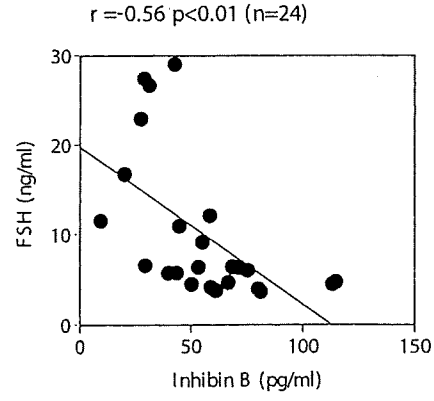

Figure 3. Relationship between plasma follicle-stimulating hormone (FSH) and inhibin B concentrations during the period from weeks 2 to 10 after the transfer from short-day (SD) to long-day (LD) photoperiods in male golden hamsters. There was an inverse correlation ($r = -0.56$, $n = 24$, $P < .01$) between these 2 hormones.

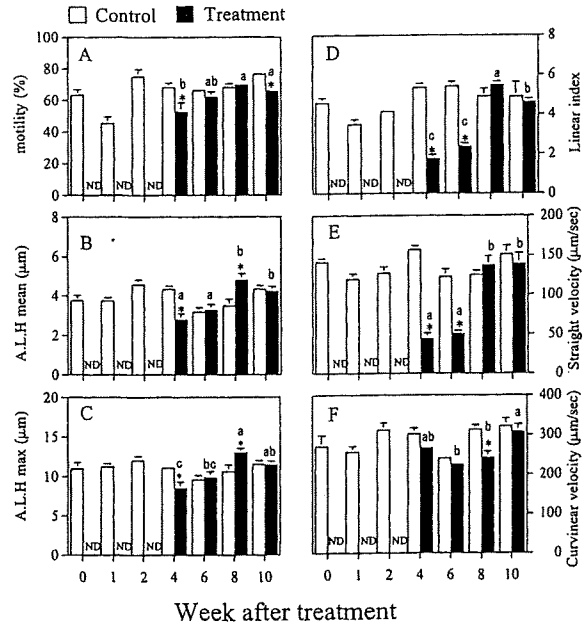

Figure 4. Epididymal sperm motility parameters: percentages of motile spermatozoa **(A)**, amplitude of lateral displacement (ALH) mean **(B)**, ALH max **(C)**, linear index **(D)**, straight velocity **(E)**, and curvilinear velocity (F) during a 10-week period after the transfer from short-day (SD) to long-day (LD) photoperiods in male golden hamsters. No sperm were detected in the cauda epididymis until 4 week of the LD photoperiod. Each point represents the mean plus or minus the standard error of the mean from 5 animals. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .0001$ compared with the control group; different characters indicate a significant difference ($P < .05$) in each graph among the treatment group.

these levels were still significantly low compared with those in the control groups; however, this was followed by further increases to the control levels by week 6. Linear index (Figure 4D) and straight velocity (Figure 4E) were still significantly ($P < .05$) low in the treatment group at weeks 4 and 6 compared with those in the control animals. These parameters recovered to those of the control levels at week 8. Curvilinear velocity (Figure 4F) abruptly recovered to normal LD levels at week 4.

Immunohistochemistry for PCNA

In order to better visualize cell types, the sections were stained with hematoxylin and eosin at weeks 0, 2, 4, 6, and 8 in the treatment group (Figure 5A, B, C, D, and E, respectively) as well as in the control group (Figure 5F). At weeks 0 and 2, the seminiferous tubules contained primarily Sertoli cells and spermatogonia, but spermatocytes and round spermatids were also occasionally seen (Figure 5A and B). The testes of animals in the treatment group at weeks 0 and 2 showed dilated seminiferous tubules with small-sized spermatogenic cells, Sertoli cells, and Leydig cells. However, there were no mature spermatids contained within the seminiferous tubules. In early recrudescence, there was an increase in the number of germ cells in the basal compartment. The increase in the cell volume of Sertoli and Leydig cells, as well as the number of germ cells in the seminiferous tubules, appeared to be time-dependent during weeks 4, 6, and 8 (Figure 5C, D, and E, respectively). The testes in animals exposed to the SD photoperiod recovered to normal LD appearance at 10 weeks.

Germ cells from the testes were positively stained for PCNA at weeks 0, 2, 4, 6, and 8 in the treatment group (Figure 5M, N, O, P, and Q, respectively) and in the control group (Figure 5R) in the male golden hamsters. PCNA was positively stained in early spermatogenic cells but not in the nuclei of Sertoli and Leydig cells at weeks 0 and 2 (Figure 5M through R). The increase in the size of immunopositive cells and in the number of spermatogenic cells was time-dependent. Relatively large nuclei of spermatogenic cells were positively stained with anti-PCNA antibody in the testes of the LD controls (Figure 5R). Sections incubated with normal mouse plasma instead of primary antibody did not show any immunopositive staining at weeks 0, 2, 4, 6, and 8 in either the treatment group (Figure 5G, H, I, J, and K, respectively) or the control group (Figure 5L).

Testes Histological Analysis

The numbers of pachytene spermatocytes and the diameter measurements of seminiferous tubules are shown in the Table. Both sets of numbers increased in a time-dependent manner during testicular recrudescence. The number of pachytene spermatocytes increased to normal LD levels at week 4 and reached peak levels at week 8. The diameter of seminiferous tubules also increased but still did not recover to normal LD levels at week 4; however, this was followed by a further increase to supracontrol levels at weeks 6 and 8.

Discussion

This is the first study that describes in detail the changes in 3 forms of inhibins—ir-inhibin, inhibin B, and inhibin pro-αC—and indirectly indicates that inhibin B affects FSH secretion during testicular recrudescence in the golden hamster. Furthermore, the results of PCNA together with the histological analysis suggest that testicular recrudescence is based on the increase in the number of spermatogenic cells rather than the increase in the number of Sertoli and Leydig cells in the testes of the male golden hamster. The present study shows that plasma FSH is rapidly elevated after the transfer of animals from an SD to an LD photoperiod and that it then begins to decline at a time when plasma inhibin B is elevated, suggesting that inhibin B is the major regulator of plasma FSH concentrations during this time. This finding is also reflected in

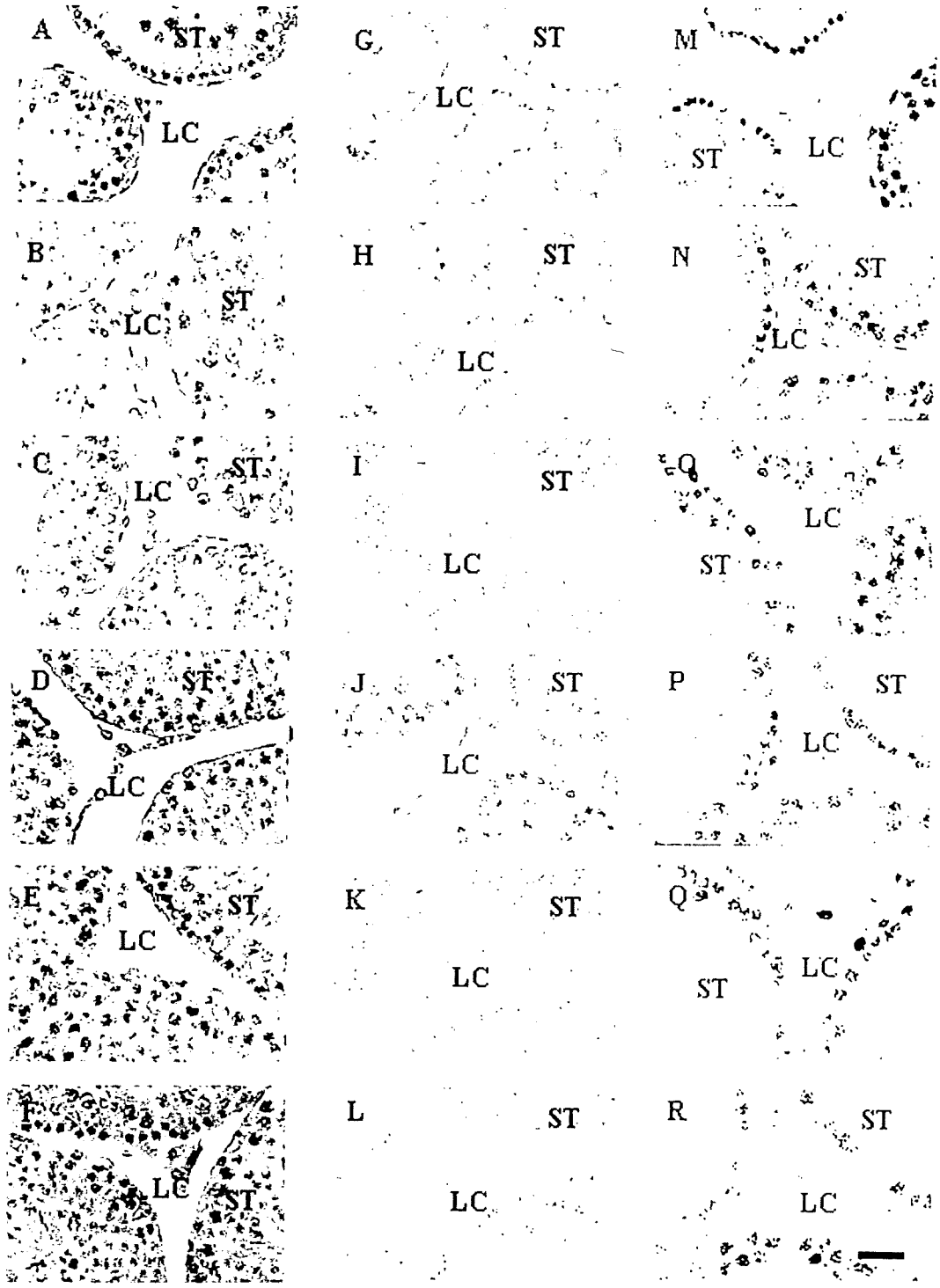

Figure 5. Immunohistochemical staining of proliferating cell nuclear antigen (PCNA) during a 10-week period after the transfer from short-day (SD) to long-day (LD) photoperiods in the testes of male golden hamsters. Germ cells at multiple stages of development were positively stained for PCNA at weeks 0 (M), 2 (N), 4 (O), 6 (P), and 8 (Q). A positive staining of PCNA in the germ cells of the control animals, which were exposed to the LD photoperiod, is also given (R). To better visualize cell types, the sections were stained with hematoxylin and eosin and are shown for weeks 0 (A), 2 (B), 4 (C), 6 (D), and 8 (E), as well as for control animals (F). Sections incubated with normal mouse plasma instead of primary antibody did not show any immunopositive staining at weeks 0 (G), 2 (H), 4 (I), 6 (J), or 8 (K); the same was true for the control animals (L). Bar = 20 μm.

*Histological analysis of spermatogenesis**

Group	Pachytene Spermatocytes	Tubular Diameter (μm)
0	13.33 ± 2.81A†	98.5 ± 3.19A
2	33.0 ± 3.15AB	166.0 ± 4.44B
4	43.4 ± 7.28BC	172.5 ± 5.93B
6	60.0 ± 6.63C	258.0 ± 5.88D
8	72.4 ± 3.47D	268.0 ± 5.97D
Control	60.6 ± 7.31C	228.25 ± 5.70C

* Shown are the means plus or minus the standard error (n = 5).
† Means with different letters in the same column are significantly different ($P < .05$; Duncan multiple range test).

a strong inverse correlation between these 2 hormones. The present study also demonstrates that gradual increases in plasma LH and testosterone occurring at the later stage of the LD photoperiod restore spermatogenesis, which has been adversely affected during exposure to the SD photoperiod.

Previous studies have suggested that in men, inhibin B regulates FSH secretion (Anawalt et al, 1996; Illingworth et al, 1996; Nachtigall et al, 1996; Seminara et al, 1996). We have also shown in our previous studies that inhibin B is the major circulating form of dimeric inhibins in the male golden hamster (Jin et al, 2001b) and in the Gottingen miniature pig (Jin et al, 2001a). Others have shown inhibin B as the major form of inhibins in other species as well, including male monkeys (Plant et al, 1997; Foppiani et al, 1999; Ramaswamy et al, 2000), male rats (Woodruff et al, 1996), and male chimpanzees (Kondo et al, 2000). It has been reported that inhibin B may have a physiological role in the development of spermatogenesis in the testes of adult male animals (Foppiani et al, 1999; Ramaswamy et al, 2000), though equine fetal testes (Tanaka et al, 2002) and ovine adult testes (McNeilly et al, 2002) secrete a large amount of inhibin A. In line with these findings, it is quite possible that there is inhibin B-FSH negative feedback during testicular recrudescence in the male golden hamster. Kirby et al (1993) did not observe any correlation between inhibin α and FSH levels during testicular recrudescence. We did not measure plasma inhibin A levels in the present study because we have shown in a previous study that plasma inhibin A is undetectable in the male golden hamster (Jin et al, 2001b).

In our present study, abrupt increases in plasma FSH levels preceded changes in plasma LH and testosterone after the animals were transferred from the SD to the LD photoperiod. Plasma FSH levels continued to rise through week 2 after transferral from the SD to the LD photoperiod, while there were gradual increases in basal plasma LH and testosterone. This result corroborates previous studies (Milette et al, 1988; Donham et al, 1994), which have reported that elevated FSH levels preceded spontaneous recrudescence of the testes in male golden hamsters. It also has been suggested that a rapid increase in plasma FSH after photostimulation is the primary signal for initiating testicular development in Djungarian hamsters (Milette et al, 1988). It was reported that the administration of exogenous FSH causes the regrowth of testes and complete spermatogenesis in photoinhibited Djungarian hamsters (Niklowitz et al, 1989). It is reasonable to suggest that FSH is important for seminiferous tubular development as well as for inhibin production by testes. On the other hand, gradual increases in basal plasma LH and testosterone levels were also observed in the present study. It is possible that the early elevation of FSH levels stimulates the induction of LH receptors in Leydig cells, thereby increasing the responsiveness of Leydig cells to endogenous and exogenous LH and thus stimulating testosterone production (Parvinen et al, 1984; Verhoeven and Cailleau, 1985). Niklowitz et al (1989) also reported that FSH alone initiated complete spermatogenesis, whereas LH alone induced full redifferentiation of Leydig cell function, resulting in increased testosterone production in the photoinhibited and hypophysectomized Djungarian hamsters. However, plasma inhibin concentrations continued to rise until week 4, during which time plasma FSH had fallen to low levels. Inhibin B production was apparently stimulated by an early rise in FSH, thus resulting in circulating levels sufficient to suppress FSH release. This finding shows further that there is a dynamic change in hormonal interrelationships.

The present study evaluated the localization of PCNA in the cell nuclei in order to identify cellular proliferating activity occurring during testicular recrudescence. Immunolocalization of PCNA has been widely used as a method for the detection of proliferating cells in tumors (Wada et al, 1993), developing tissues (Casasco et al, 1993), and testes (Schlatt and Weinbauer, 1994). In the present study, we have identified PCNA as being localized to the nuclei of early spermatogenic cells; in addition, we have found that the number and size of these immunopositive spermatogenic cells appear to increase in a time-dependent manner. In contrast, nuclei of both Leydig and Sertoli cells were not stained for PCNA. This result agrees with previous findings that both Leydig cells (Hikim et al, 1988; Sinha Hikim et al, 1993) and Sertoli cells (Hikim et al, 1988) increased in volume but not in number during testicular recrudescence in the male golden hamster. In line with these results, Liang et al (2001) reported that, in male monkeys, PCNA-immunopositive spermatogenic cells were increased in an age-dependent manner and that the positive staining for PCNA in Sertoli cell nuclei was observed in the testes of animals from the neonatal to the pubertal stage but not in adult animals. Together with the present histological analysis (shown in the Table), it is likely that the abrupt increase in plasma FSH and the steady increase in plasma LH, which occurred at an early stage of testicular recrudescence in the

golden hamster, were responsible for the increased volume of the Sertoli and Leydig cells. This rise in plasma FSH and LH levels possibly increases Leydig cell capacity to produce an increased amount of inhibins, because Leydig cells are the major source of inhibin B in the testes of the golden hamster (Jin et al, 2001b).

Although photoperiod-induced testicular recrudescence appears to begin at an early stage in the golden hamster, we could not detect any sperm until 2 weeks after transferral to the LD photoperiod. This suggests that the first wave of spermatogenesis apparently requires approximately 1 to 2 weeks after the transfer of animals from the SD to the LD photoperiod. It should be noted, however, that although sperm were detected in an animal in the treatment group at week 2, the profiles of sperm motility parameters were very low (data not shown). It should also be noted that in other animals, although sperm were present in the cauda epididymis after 4 weeks of normal LD exposure, in general, none of the parameters returned to normal values until about weeks 6 to 8.

In conclusion, the present results demonstrate the existence of a negative feedback of inhibin B on FSH secretion in male golden hamsters after transferral from an SD to an LD photoperiod. This feedback appears to begin functioning at an early stage of photoperiod-induced spontaneous testicular recrudescence. Plasma FSH as a primary signal, in combination with other reproductive hormones such as inhibin B, is likely to be the stimulus for the proliferation of various cells, leading to the reestablishment of the spermatogenic process.

Acknowledgments

We are grateful to the Rat Pituitary Hormone Distribution Program, the National Institute of Diabetes and Digestive and Kidney Diseases (Bethesda, Md), and Dr A. F. Parlow for providing RIA materials of rat LH and FSH. We are also grateful to Dr G. D. Niswender (Animal Reproduction and Biotechnology Laboratory, Colorado State University, Fort Collins, Colo) for providing antiserum to testosterone (GDN 250) and to Dr T. Kohsaka (Laboratory of Animal Reproduction, Faculty of Agriculture, Shizuoka University) for his valuable suggestion on sperm motion analyses.

References

Anawalt BD, Bebb RA, Matsumoto AM, Groome NP, Illingworth PJ, McNeilly AS, Bremner WJ. Serum inhibin B levels reflect Sertoli cell function in normal men and men with testicular dysfunction. *J Clin Endocrinol Metab.* 1996;81:3341–3345.

Bast JD, Greenwald GS. Serum profiles of follicle-stimulating hormone, luteinizing hormone and prolactin during the estrous cycle of the hamster. *Endocrinology.* 1974;94:1295–1299.

Berndtson WE, Desjardins C. Circulating LH and FSH levels and testicular function in hamsters during light deprivation and subsequent photoperiodic stimulation. *Endocrinology.* 1974;95:195–205.

Bravo R, Macdonald-Bravo H. Existence of two populations of cyclin/proliferating cell nuclear antigen during the cell cycle: association with DNA replication sites. *J Cell Biol.* 1987;105:1549–1554.

Casasco A, Giordano M, Danova M, Casasco M, Icaro Cornaglia A, Calligaro A. PC10 monoclonal antibody to proliferating cell nuclear antigen as probe for cycling cell detection in developing tissues. A combined immunocytochemical and flow cytometric study. *Histochemistry.* 1993;99:191–199.

Donham RS, Palacio E, Stetson MH. Dissociation of the reproductive and prolactin photoperiodic responses in male golden hamsters. *Biol Reprod.* 1994;51:366–372.

Foppiani L, Schlatt S, Simoni M, Weinbauer GF, Hacker-Klom U, Nieschlag E. Inhibin B is a more sensitive marker of spermatogenetic damage than FSH in the irradiated non-human primate model. *J Endocrinol.* 1999;162:393–400.

Garrido MC, Cordell JL, Becker MH, Key G, Gerdes J, Jones M, Gatter KC, Mason DY. Monoclonal antibody JC1: new reagent for studying cell proliferation. *J Clin Pathol.* 1992;45:860–865.

Gay VL, Kerlan JT. Serum LH and FSH following passive immunization against circulating testosterone in the intact male rat and in orchidectomized rats bearing subcutaneous silastic implants of testosterone. *Arch Androl.* 1978;1:257–266.

Goldman BD, Matt KS, Roychoudhury P, Stetson MH. Prolactin release in golden hamsters: photoperiod and gonadal influences. *Biol Reprod.* 1981;24:287–292.

Hamada T, Watanabe G, Kokuho T, Taya K, Sasamoto S, Hasegawa Y, Miyamoto K, Igarashi M. Radioimmunoassay of inhibin in various mammals. *J Endocrinol.* 1989;122:697–704.

Hikim AP, Bartke AJ, Russell LD. The seasonal breeding hamster as a model to study structure-function relationships in the testis. *Tissue Cell.* 1988;20:63–78.

Hofstadter F, Knuchel R, Ruschoff J. Cell proliferation assessment in oncology. *Virchows Arch.* 1995;427:323–341.

Illingworth PJ, Groome NP, Byrd W, Rainey WE, McNeilly AS, Mather JP, Bremner WJ. Inhibin-B: a likely candidate for the physiologically important form of inhibin in men. *J Clin Endocrinol Metab.* 1996; 81:1321–1325.

Jin W, Arai KY, Herath CB, et al. Inhibins in the male Gottingen miniature pig: Leydig cells are the predominant source of inhibin B. *J Androl.* 2001a;22:953–960.

Jin W, Wada S, Arai KY, et al. Testicular secretion of inhibin in the male golden hamster (*Mesocricetus auratus*). *J Androl.* 2001b;22:207–211.

Kirby JD, Jetton AE, Ackland JF, Turek FW, Schwartz NB. Changes in serum immunoreactive inhibin-alpha during photoperiod-induced testicular regression and recrudescence in the golden hamster. *Biol Reprod.* 1993;49:483–488.

Kondo M, Udono T, Jon W, et al. Changes in plasma concentrations of inhibin A and inhibin B throughout sexual maturation in the male chimpanzee. *Endocr J.* 2000;47:707–714.

Lerchl A, Nieschlag E. Interruption of nocturnal pineal melatonin synthesis in spontaneous recrudescent Djungarian hamsters (*Phodopus sungorus*). *J Pineal Res.* 1992;13:36–41.

Liang JH, Sankai T, Yoshida T, Yoshikawa Y. Immunolocalization of proliferating cell nuclear antigen (PCNA) in cynomolgus monkey (*Macaca fascicularis*) testes during postnatal development. *J Med Primatol.* 2001;30:107–111.

Ling N, Ying SY, Ueno N, Esch F, Denoroy L, Guillemin R. Isolation and partial characterization of a Mr 32 000 protein with inhibin activity from porcine follicular fluid. *Proc Natl Acad Sci USA.* 1985;82: 7217–7221.

Matt KS, Stetson MH. Comparison of serum hormone titers in golden hamsters during testicular growth induced by pinealectomy and photoperiodic stimulation. *Biol Reprod.* 1980;23:893–898.

McNeilly AS, Souza CJH, Baird DT, Swanston IA, McVerry J, Crawford J, Cranfield M, Lincoln GA. Production of inhibin A not B in rams:

changes in plasma inhibin A during testis growth, and expression of inhibin/activin subunit mRNA and protein in adult testis. *Reproduction.* 2002;123:827–835.

Meredith JM, Turek FW, Levine JE. Effects of gonadotropin-releasing hormone pulse frequency modulation on the reproductive axis of photoinhibited male Siberian hamsters. *Biol Reprod.* 1998;59:813–819.

Milette JJ, Schwartz NB, Turek FW. The importance of follicle-stimulating hormone in the initiation of testicular growth in photostimulated Djungarian hamsters. *Endocrinology.* 1988;122:1060–1066.

Miyamoto K, Hasegawa Y, Fukuda M, Nomura M, Igarashi M, Kangawa K, Matsuo H. Isolation of porcine follicular fluid inhibin of 32K daltons. *Biochem Biophys Res Commun.* 1985;129:396–403.

Morita T, Usuda N, Hanai T, Nagata T. Changes of colon epithelium proliferation due to individual aging with cyclin proliferating cell nuclear antigen (PCNA/cyclin) immunostaining compared to [3H]-thymidine radioautography. *Histochemistry.* 1994;101:13–20.

Nachtigall LB, Boepple PA, Seminara SB, Khoury RH, Sluss PM, Lecain AE, Crowley WF Jr. Inhibin B secretion in males with gonadotropin-releasing hormone (GnRH) deficiency before and during long-term GnRH replacement: relationship to spontaneous puberty, testicular volume, and prior treatment—a clinical research center study. *J Clin Endocrinol Metab.* 1996;81:3520–3525.

Niklowitz P, Khan S, Bergmann M, Hoffmann K, Nieschlag E. Differential effects of follicle-stimulating hormone and luteinizing hormone on Leydig cell function and restoration of spermatogenesis in hypophysectomized and photoinhibited Djungarian hamsters (*Phodopus sungorus*). *Biol Reprod.* 1989;41:871–880.

Parvinen M, Nikula H, Huhtaniemi I. Influence of rat seminiferous tubules on Leydig cell testosterone production in vitro. *Mol Cell Endocrinol.* 1984;37:331–336.

Plant TM, Padmanabhan V, Ramaswamy S, McConnell DS, Winters SJ, Groome N, Midgley AR Jr, McNeilly AS. Circulating concentrations of dimeric inhibin A and B in the male rhesus monkey (*Macaca mulatta*). *J Clin Endocrinol Metab.* 1997;82:2617–2621.

Ramaswamy S, Marshall GR, McNeilly AS, Plant TM. Dynamics of the follicle-stimulating hormone (FSH)-inhibin B feedback loop and its role in regulating spermatogenesis in the adult male rhesus monkey (*Macaca mulatta*) as revealed by unilateral orchidectomy. *Endocrinology.* 2000;141:18–27.

Rivier J, Spiess J, McClintock R, Vaughan J, Vale W. Purification and partial characterization of inhibin from porcine follicular fluid. *Biochem Biophys Res Commun.* 1985;133:120–127.

Robertson DM, Foulds LM, Leversha L, Morgan FJ, Hearn MT, Burger HG, Wettenhall RE, de Kretser DM. Isolation of inhibin from bovine follicular fluid. *Biochem Biophys Res Commun.* 1985;126:220–226.

Schlatt S, Weinbauer GF. Immunohistochemical localization of proliferating cell nuclear antigen as a tool to study cell proliferation in rodent and primate testes. *Int J Androl.* 1994;17:214–222.

Seminara SB, Boepple PA, Nachtigall LB, Pralong FP, Khoury RH, Sluss PM, Lecain AE, Crowley WF Jr. Inhibin B in males with gonadotropin-releasing hormone (GnRH) deficiency: changes in serum concentration after shortterm physiologic GnRH replacement—a clinical research center study. *J Clin Endocrinol Metab.* 1996;81:3692–3696.

Sharpe RM, Turner KJ, McKinnell C, Groome NP, Atanassova N, Millar MR, Buchanan DL, Cooke PS. Inhibin B levels in plasma of the male rat from birth to adulthood: effect of experimental manipulation of Sertoli cell number. *J Androl.* 1999;20:94–101.

Shivji KK, Kenny MK, Wood RD. Proliferating cell nuclear antigen is required for DNA excision repair. *Cell.* 1992;69:367–374.

Simpson SM, Follett BK, Ellis DH. Modulation by photoperiod of gonadotrophin secretion in intact and castrated Djungarian hamsters. *J Reprod Fertil.* 1982;66:243–250.

Sinha Hikim AP, Chandrashekar V, Bartke A, Russell LD. Sentinels of Leydig cell structural and functional changes in golden hamsters in early testicular regression and recrudescence. *Int J Androl.* 1993;16:324–342.

Steel RGD, Torrie JH. *Principles and Procedures of Statistics.* New York, NY: McGraw-Hill; 1960.

Tanaka Y, Taniyama H, Tsunoda N, et al. The testis as a major source of circulating inhibins in the male equine fetus during the second half of gestation. *J Androl.* 2002;23:229–236.

Taya K, Watanabe G, Sasamoto S. Radioimmunoassay for progesterone, testosterone, and estradiol-17β using ^{125}I-iodohistamine radioligands. *Jpn J Anim Reprod.* 1985;31:186–197.

Turek FW, Elliott JA, Alvis JD, Menaker M. Effect of prolonged exposure to nonstimulatory photoperiods on the activity of the neuroendocrine-testicular axis of golden hamsters. *Biol Reprod.* 1975;13:475–481.

Vale W, Rivier J, Vaughan J, McClintock R, Corrigan A, Woo W, Karr D, Spiess J. Purification and characterization of an FSH releasing protein from porcine ovarian follicular fluid. *Nature.* 1986;321:776–779.

Verhoeven G, Cailleau J. A factor in spent media from Sertoli-cell–enriched cultures that stimulates steroidogenesis in Leydig cells. *Mol Cell Endocrinol.* 1985;40:57–68.

Wada T, Shimabukuro T, Kurisu H, et al. Expression of proliferating cell nuclear antigen in superficial bladder cancer—application to paraffin-embedded tissue sections. *Hinyokika Kiyo.* 1993;39:313–319.

Woodruff TK, Besecke LM, Groome N, Draper LB, Schwartz NB, Weiss J. Inhibin A and inhibin B are inversely correlated to follicle-stimulating hormone, yet are discordant during the follicular phase of the rat estrous cycle, and inhibin A is expressed in a sexually dimorphic manner. *Endocrinology.* 1996;137:5463–5467.

Xiong Y, Zhang H, Beach D. D type cyclins associate with multiple protein kinases and the DNA replication and repair factor PCNA. *Cell.* 1992;71:505–514.