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Effects of Bilateral Efferent Duct Ligation on Sperm Motility and Secretion of FSH, LH, Inhibin, and Testosterone in Adult Male Rats

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Effects of bilateral efferent duct ligation (EDL) on sperm motility and testicular endocrinology were investigated in adult male rats. Bilateral EDL was created surgically in adult male rats (EDL group) and sham-operated rats were used as control (control group). Five rats from each group were killed on d 3, 5, 7, 14, and 35 after the surgery. The sperm motility parameters were determined by a computer-assisted sperm analysis system using sperm collected from the cauda epididymis. Concentrations of spermatozoa in epididymis and testis were counted. The motility of sperm decreased remarkably in EDL rats compared with controls on 5 d after the operation. Four sperm motility parameters—straight velocity (VSL), deviation of the sperm head from the mean trajectory (ALH, mean), the maximum amplitude of lateral head displacement (ALH, max) and curvilinear velocity (VCL)—increased on 3 d after the operation, and followed by a subsequent decline 5 and 7 d later. Concentrations of sperm significantly decreased in both testes and epididymis from 3 and 5 d after the operation. Plasma concentrations of FSH and LH increased significantly in EDL rats from 5 and 7 d after the operation, whereas plasma concentrations of immunoreactive (ir)-inhibin, inhibin B, and testosterone decreased. Testicular content of ir-inhibin showed an initial increase on 3 d after the operation, followed by a subsequent decline to levels significantly below controls by d 7 postoperation. On the other hand, testicular contents of testosterone were significantly higher in the EDL group than the control group on d 7–35 after the operation, whereas circulating levels of testosterone remained low. In the EDL testes, marked degenerative changes in the Sertoli cells and spermatogonia were observed, whereas Leydig cells showed clear hyperplasia. These results demonstrated that bilateral EDL induced a rapid reduction of sperm motility parameters during a short time. Present results also suggest that EDL first induces impairment of Sertoli cells function and this leads to reduction of sperm activity and secretion of inhibins. On the other hand, circulating levels of testosterone reduced after EDL and this leads to hypersecretion of LH. A large amount of LH resulted in a stimulation of Leydig cells hyperplasia.

Key Words: Inhibin B; testosterone; Sertoli cell; Leydig cell; sperm motion.

Introduction

Spermatogenesis is a unique process of continuing differentiation because unlike other vital processes such as hematopoiesis, the DNA content of the resulting product is precisely one half of the progenitor stem cell. During the spermatogenic process, which is unique and fixed in the time frame for each species, numerous cellular interactions in the triangle involving Sertoli cells, germ cells, and the Leydig cells allow for attachment and growth of the germ cells into haploid sperm. Sertoli cells were thought to provide the scaffolding necessary for the anchoring and nourishment of the germ cell. Thus, in this process the continuously transforming germ cell in its various stages is transported from the basal lamina toward the lumen. It has been known that efferent ducts ligation (EDL) causes retention of the fluid secreted in the testis, leading initially to distention of the testis, degeneration of the germinal epithelium and

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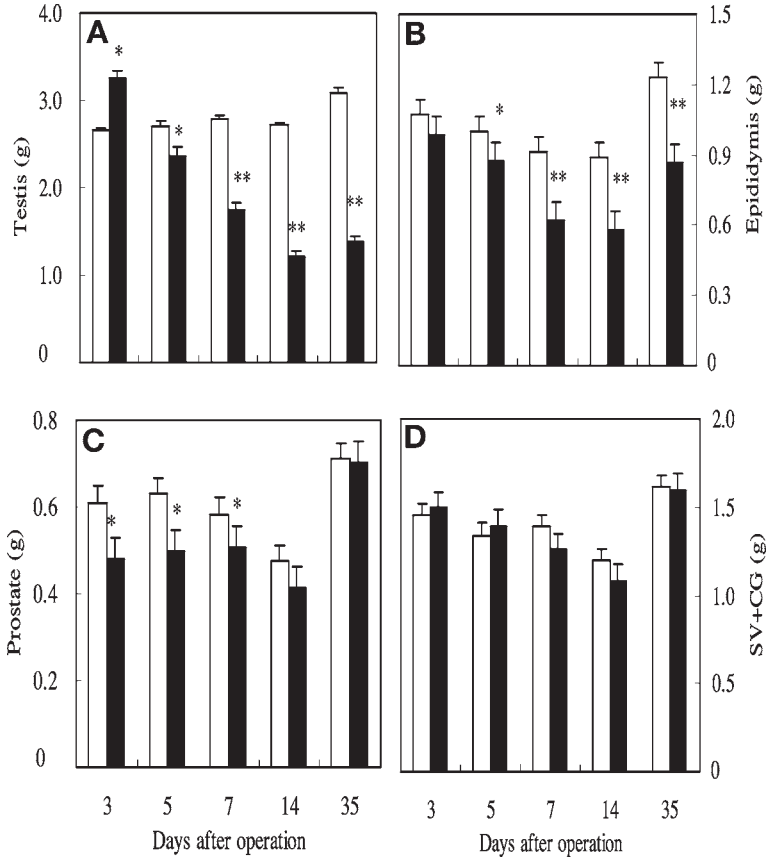

Fig. 1. Weight of testis (**A**), epididymis (**B**), prostate (**C**), seminal vesicle and coagulating gland complex (**D**) in adult male rats after bilateral efferent duct ligation (solid bar) or sham-operation (open bar). Each point represents the mean ± SEM of five animals. *$p < 0.05$; **$p < 0.001$ compared with the control value.

cessation of spermatogenesis *(1,2)*. EDL of the mammalian testis results in an initial accumulation of fluid and secretory products in the seminiferous tubules and subsequent decline in the testicular weight and atrophy of the seminiferous epithelium *(2–4)*. In the field of animal production, spermic granuloma is a serious problem especially in male domestic animals, such as goats, cattle, and sheep. Large spermic granulomas in the efferent ductules are a major cause of sterility in male animals *(5)*. However, influence of spermic granulomas on testicular function, especially sperm motility, sperm motion, and testicular endocrinology, are unclear. The present study was undertaken to determine the effect of experimentally induced EDL on sperm motility characteristics and testicular endocrinology.

Results

Changes in Weight of Reproductive Organs

EDL resulted in an initial increase in the weight of the testes 3 d postoperation compared with the control group (Fig. 1A). Thereafter, from d 5 to 35 postoperation, the testicular weight significantly decreased compared with the control group. The testicular weight of the EDL rat decreased to about 50% of the control group 14 and 35 d postoperation. The weight of epididymis in the EDL rat significantly decreased on d 5 postoperation onward compared with the control group (Fig. 1B). The prostate weight significantly decreased from d 3 to d 7 postoperation (Fig. 1C). On the other hand, there was no significant change in the weight of seminal vesicles between the EDL and control groups (Fig. 1D).

Changes in Sperm Motility Parameters and Sperm Head Count

Data from computer-assisted sperm motility analysis are shown in Fig. 2. No sperm was available in the cauda epididymidis for motility parameter analysis after d 14 in the EDL group and therefore results after d 14 are not shown. EDL significantly reduced sperm motility after 5 d as compared with the control group. Seven days after EDL, sperm motility was about 75% of the control group (Fig. 2A). There were significant increases in four sperm motility parameters, the mean straight velocity (VSL), ALH mean, ALH max, and curvilinear velocity (VCL) 3 d after EDL. Thereafter, the four parameters decreased 5 and 7 d later in the EDL group (Figs. 2B,C,E,F). There was no significant change in the linearity index between EDL and the control groups (Fig. 2D). Moreover, EDL resulted in sig-

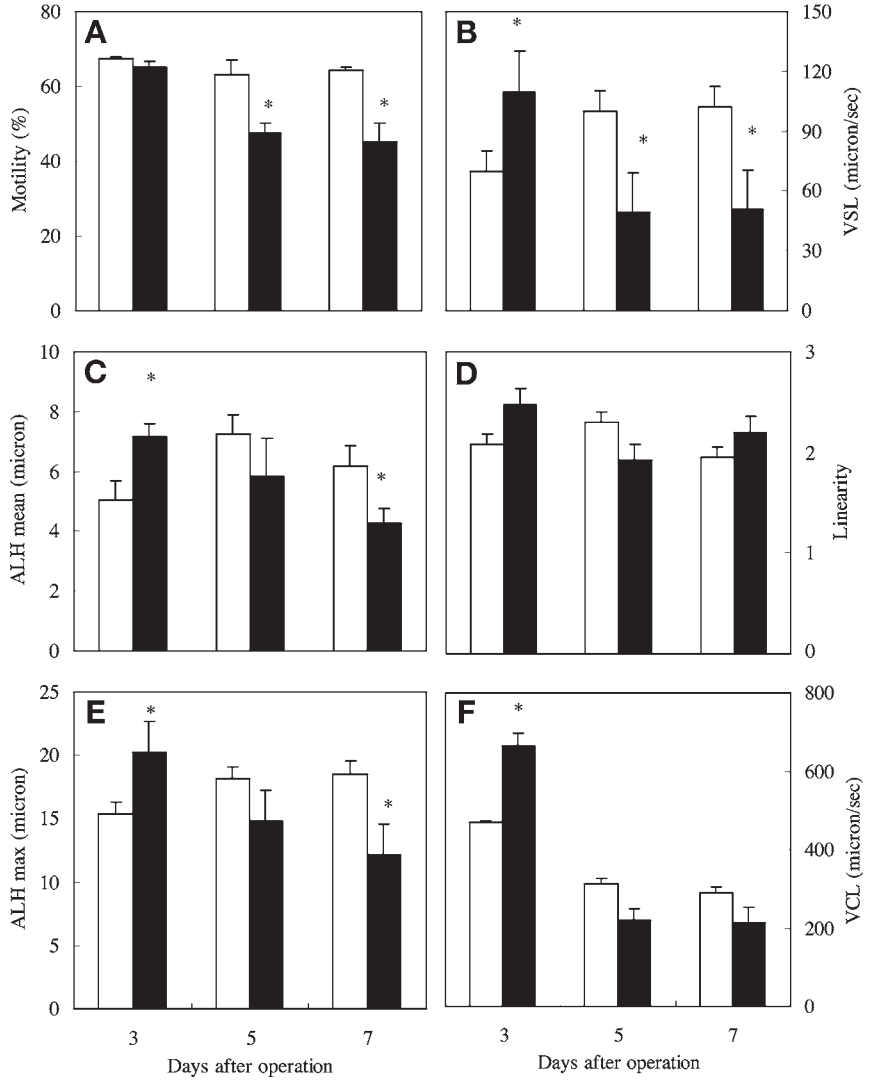

Fig. 2. Changes in epididymal sperm motility parameters: percentages of motile spermatozoa (**A**), straight velocity (VSL) (**B**), amplitude of lateral displacement (ALH) mean (**C**), linearity index (**D**), amplitude of lateral displacement (ALH) max (**E**), and curvilinear velocity (VCL) (**F**) during days after bilateral efferent duct ligation (solid bar) or sham-operation (open bar). Each point represents the mean ± SEM of five animals. *$p < 0.05$ compared with the control value.

nificant decreases in sperm head count in testis and epididymis after 3 and 5 d onward as compared with the control group (Figs. 3A,B).

Morphology of Testes

Morphology of testes in the rats after bilateral EDL is shown in Fig. 4. No changes of morphology of testes were observed in the control from 3 to 35 d after the sham operation, and therefore only the result of d 3 after the sham operation is presented (Fig. 4A). At d 3 postoperation, the number and morphology of spermatogonia in the EDL rat were similar to those for controls. However, severe cytoplasmic vacuolization was observed in the basal cytoplasm of Sertoli cells, and some tubules showed very large vacuoles, whereas others presented multiple small vesicles in the EDL testes on d 3 postoperation (Fig. 4B). These vacuoles increased at d 5 postoperation (Fig. 4C). A few multinucleated giant cells were seen in the lumen of some tubules, and their clear characteristics were similar to those of abnormal isolated round spermatids (Fig. 4C). In addition, spermatocytes and round spermatids were massively lost into the lumen of the seminiferous tubules from d 5 postoperation (Figs. 4C–F). The round spermatids and elongating spermatids were not observed in the lumen of the seminiferous tubules on d 14 and 35 (Fig. 4E,F). EDL resulted in a significant decrease in seminiferous tubules diameters from d 5 after the operation (Fig. 5). On the other hand, interstitial cell hyperplasia was observed from d 7 postoperation (Figs. 4D–F).

Immunohistochemistry for PCNA and the 3β-Hydroxysteroid Dehydrogenase (3β-HSD)

In order to demonstrate hyperplasia of Leydig cells in testes of rats with bilateral EDL, sections were immuno-

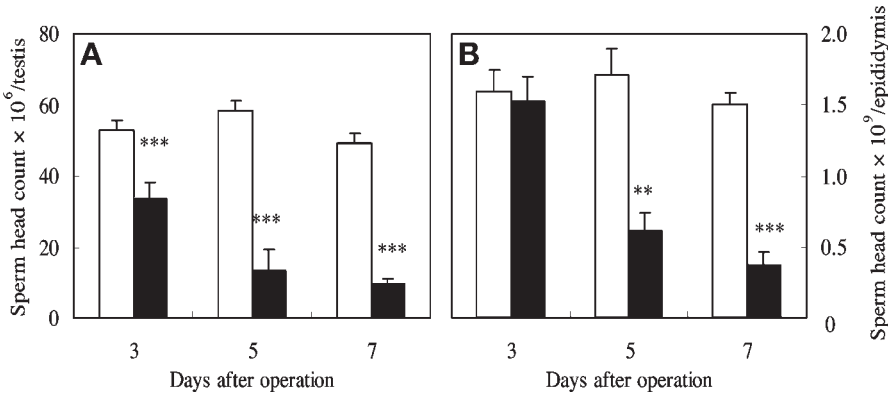

Fig. 3. Sperm count in testis (**A**) and caudal epididymis (**B**) during days after induced bilateral efferent duct ligation (solid bar) or sham-operation (open bar). Each point represents the mean ± SEM of five animals. $^{**}p < 0.001$, $^{***}p < 0.0001$ compared with the control value.

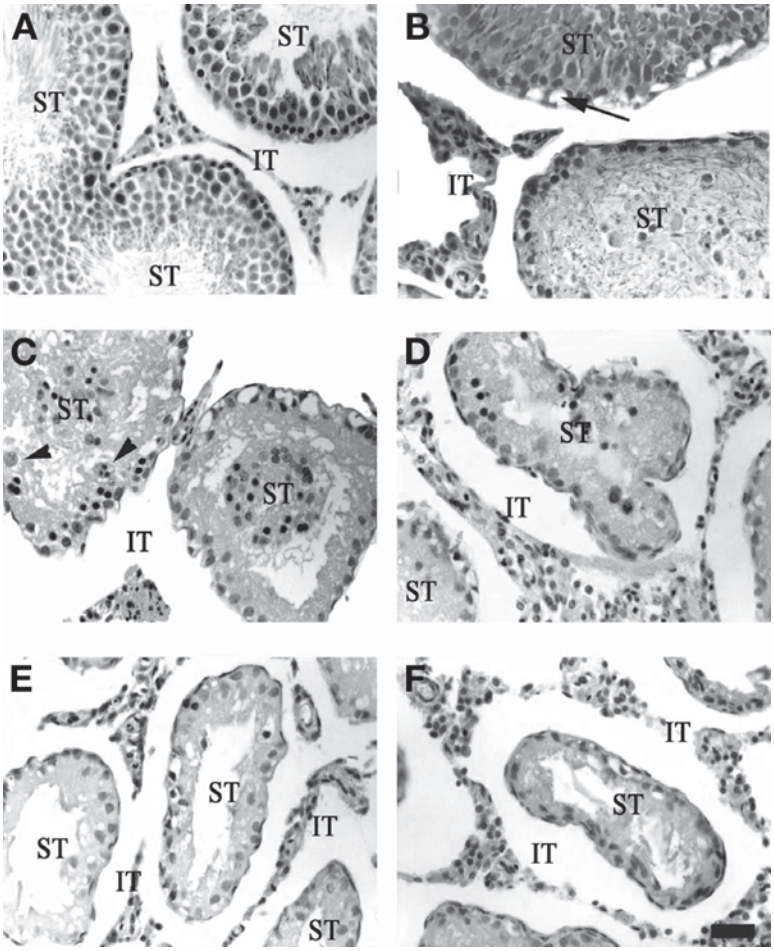

Fig. 4. Morphological changes of testes in adult male rats after bilateral efferent duct ligation. Panel **A** is from d 3 of the sham operated rats, and **B**, **C**, **D**, **E**, and **F** are from the ligated rats on d 3, 5, 7, 14, and 35 postoperation, respectively. All sections represent testes stained with hematoxylin–eosin. (**B**) 3 d. All germ cells, usually present at this stage, were present and displayed a normal appearance. However Sertoli cells show a marked vacuolization (arrow). (**C**) 5 d. Elongating spermatids were not observed. Spermatocytes and round spermatids were massively lost. Smaller multinucleated cells present in the seminiferous tubules (arrowheads) (**D,E,F**) 7, 14, and 35 d. At d 7 after efferent duct ligation most tubules show a reduction in the tubular diameter and various degrees of degeneration. Leydig cells hyperplasia from d 7 after operation. IT: interstitial cells, ST: seminiferous tubule. Bar = 30 μm. All sections are shown at the same magnification.

stained for proliferating cell nuclear antigen (PCNA) and 3β-HSD. Results concerning with immunohistochemistry for PCNA and 3β-HSD are shown in Fig. 6.

Interstitial cells and germ cells but not Sertoli cells from the testes were positively stained for PCNA from d 3 to 35 in the EDL and control groups. The number of PCNA-posi-

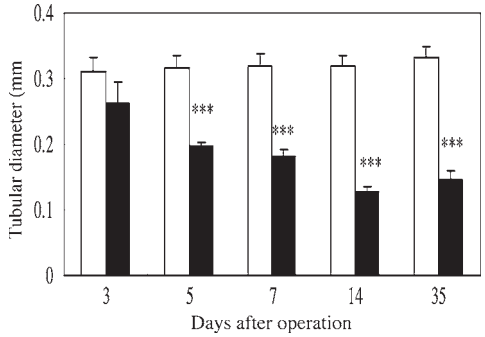

Fig. 5. Changes in seminiferous tubular diameters during days after induced bilateral efferent duct ligation (solid bar) or sham-operation (open bar). Each point represents the mean ± SEM of five animals. ***$p < 0.0001$ compared with the control value.

tive interstitial cells was increased from d 7 after ligation (Figs. 6D–F). In addition, the number of interstitial cells positively stained with 3β-HSD was also increased in the EDL group from d 7 postoperation (Figs. 6J–L). These results were in agreement with the hematoxylin-eosin (HE) staining for observations of general histology (Fig. 5).

Plasma and Testicular Hormones

Plasma concentrations of LH remained basal until d 5 after the operation, and were followed by a gradual increase in the EDL group. Plasma concentrations of LH in the EDL group were significantly high on d 7, 14, and 35 after surgery as compared with the control group (Fig. 7A). Plasma concentrations of testosterone were significantly lower in the EDL group at 14 and 35 d after the operation as compared with the control group (Fig. 7C), showing the negative correlation with circulating LH. However, testicular contents of testosterone were significantly higher in the EDL group than in the control group on d 7, 14 and 35 post-operation (Fig. 8A). This correlated well with the increase number of Leydig cells in the testis of the EDL rats (Figs. 5 and 6).

Plasma concentrations of FSH significantly increased in the EDL group on d 5 after the operation onward as compared with the control group (Fig. 7B). In contrast, plasma concentrations of ir-inhibin gradually declined in the EDL group from d 5 to d 35 after the operation (Fig. 7D). Changes in plasma concentrations of inhibin B were parallel with ir-inhibin (Fig. 9). A similar pattern of changes in testicular contents of ir-inhibin was observed except d 3 after the operation. There was a significant increase in testicular contents of ir-inhibin in the EDL group on d 3 after surgery as compared with the control group (Fig. 8B).

Discussion

The present study clearly demonstrated that bilateral EDL in adult rats caused acute changes of sperm motility parameters in the epididymis within a short period following the operation, although sperm motility in the EDL group still remained about 75% of the control until 7 d after the operation. Thereafter, on d 14 after the operation, no sperm was available in the cauda epididymis. The characterization and regulation of the epididymal protein microenvironment has been of interest for many years *(6–9)*, and it has been established that some of these proteins are important for sperm maturation and sperm function at the site of fertilization *(10, 11)*. EDL alters concentrations of apolipoprotein J (clusterin or sulfated glycoprotein-2), low-density lipoprotein receptor–related protein-2 (LRP-2), gamma-glutamyl transpeptidase (GGT), and phospholipid hydroperoxide glutathione peroxidase (PHGPX) in epididymis *(12–14)*. These changes may affect the sperm maturation and motility. The rapid loss of these proteins from sperm and reduced protein secretion from epididymis after EDL suggests that this surgical intervention may affect spermatozoa residing within the epididymis *(15,16)*. In addition, motility in sperm is mainly controlled by hormones, ions (K^+, Ca^{2+}, etc.), and pH *(17, 18)*. Furthermore, EDL induced a similar pattern of apoptosis in the initial segment of the epididymis as that seen after orchidectomy. Androgen replacement therapy after orchidectomy demonstrated that apoptosis in the caput, corpus, and cauda epididymidis was androgen dependent. However, androgens alone could not completely prevent apoptosis in the initial segment of the epididymis *(19)*. In the present study, there was a significant decrease in the epididymis weight within 5 d after the operation (Fig. 1B). Therefore, the decrease in sperm motility in EDL rats might be attributed to impairment in the epididymal function.

The present results indicated that bilateral EDL induced different changes in Sertoli cells and Leydig cells. These results demonstrated that EDL induced clear degenerative changes in testicular morphology, such as vacuolization of Sertoli cells and loss of spermatocytes or round spermatids, whereas the number of the Leydig cells increased. In addition, the present study showed a clear disruption of the feedback effect between FSH and inhibins. The plasma inhibin B level is an accurate marker of the function of Sertoli cells *(20,21)* and is useful in the clinical evaluation of male fertility. Because Sertoli cell function in several mammals is reflected by plasma concentrations of inhibin B *(20, 22–25)* and androgen-binding protein *(26,27)*, in the present study, the low circulating inhibin B levels clearly indicate impairment of Sertoli cell function. Moreover, after bilateral EDL; the testicular ir-inhibin content showed an initial increase on 3 d after the operation, followed by a subsequent decline. The decline in testicular ir-inhibin parallels with the increase in degenerative changes of Sertoli cells in the seminiferous tubule after EDL. An inverse relationship between circulating FSH and inhibins was clearly observed in the EDL group. These results clearly indicated that circulating levels of inhibins significantly decreased after bilateral EDL; this in turn lead to an elevation of circulating FSH by cancelling the negative feedback effect by inhibins.

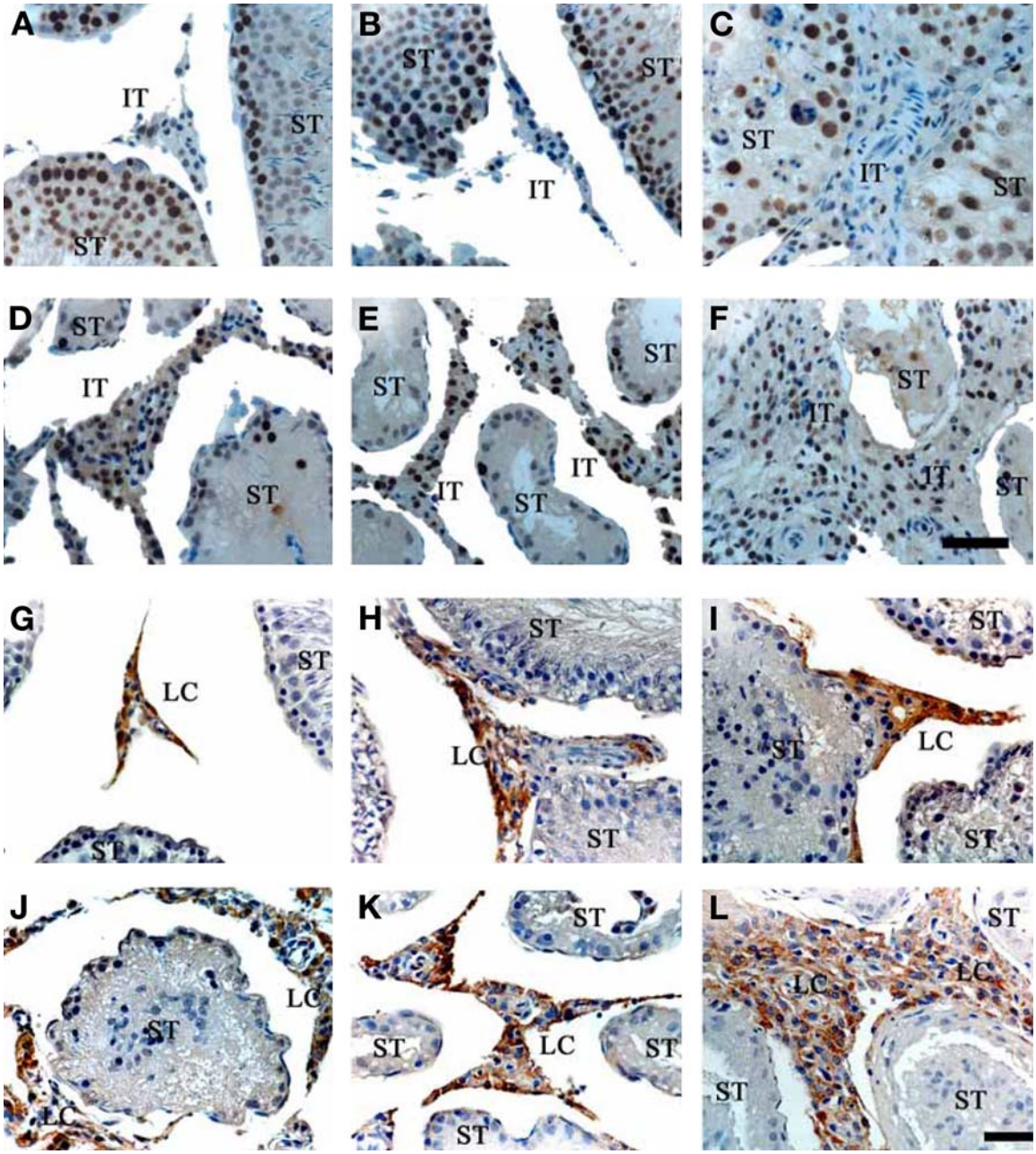

Fig. 6. Immunohistochemical staining of proliferating cell nuclear antigen (PCNA) and 3β-hydroxysteroid dehydrogenase (3β-HSD) in Leydig cells of testes from controls (**A** and **G**) and 3, 5, 7, 14, and 35 d after operation (**B–F** and **H–L**) in adult rats. Panels (**B,C,D, E,F**) and (**H,I,J,K,L**) show immunohistochemical localization of PCNA and 3β-HSD, respectively. An increase in the number of immunopositive interstitial cells from 7 d after operation, the intensity of the immunohistochemical signal for PCNA and 3β-HSD appear in panels (**D,E,F**) and (**J,K,L**), respectively. IT: interstitial cells; ST: seminiferous tubule; LC: Leydig cell. Bar = 30 μm.

The present study demonstrated that testicular contents of testosterone increased after EDL. In addition, confined Leydig cells showed hyperplasia from d 7 after bilateral EDL in the adult rats. High levels of LH probably induced Leydig cell hyperplasia. The present results were further supported by findings of Risbridger et al. *(28)*, who demonstrated an increase in Leydig cell size and decrease in serum testosterone after EDL. The present study, using immunohistochemistry for PCNA and the 3β-HSD, demonstrated that the number of Leydig cells were markedly increased. The increased testicular level of testosterone could be attributed to hyperplasia of Leydig cells. However, EDL rats showed reduced circulating levels of testosterone despite increased testicular contents of testosterone. This might be attributed to failure of testosterone to release blood vessels. The failure of testosterone secretion to rise

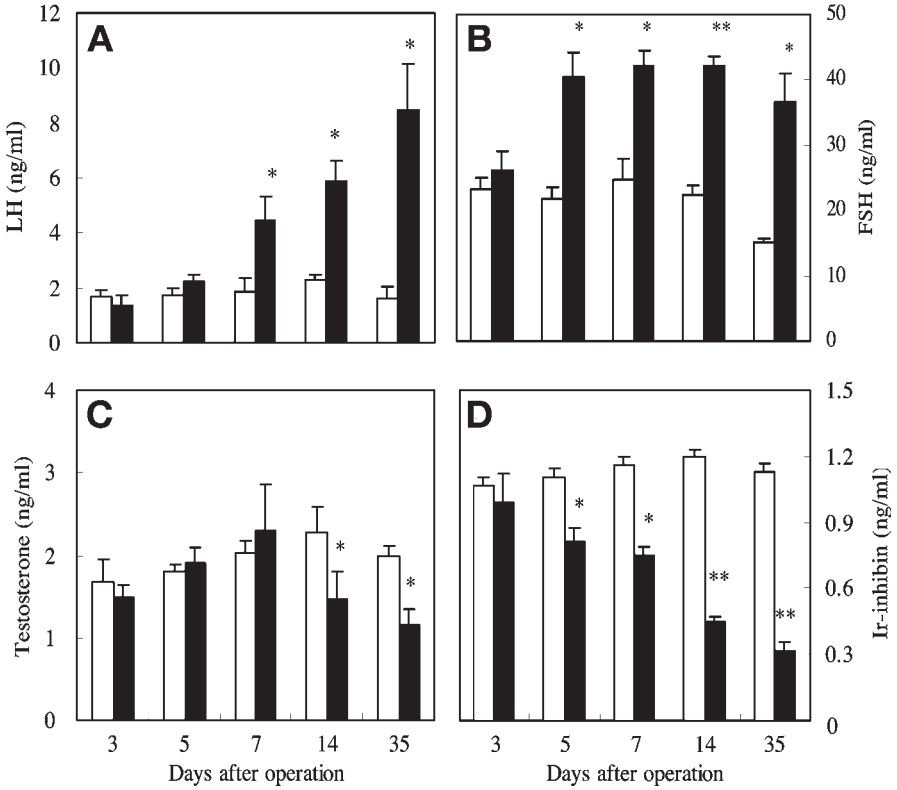

Fig. 7. Changes in plasma concentrations of (**A**) luteinizing hormone (LH), (**B**) follicle-stimulating hormone (FSH), (**C**) testosterone, and (**D**) immunoreactive (ir-) inhibin in male rats after bilateral efferent duct ligation (solid bar) or sham-operation (open bar) in rats. Each value represents the mean ± SEM of five animals. *$p < 0.05$; **$p < 0.001$ compared with the control value.

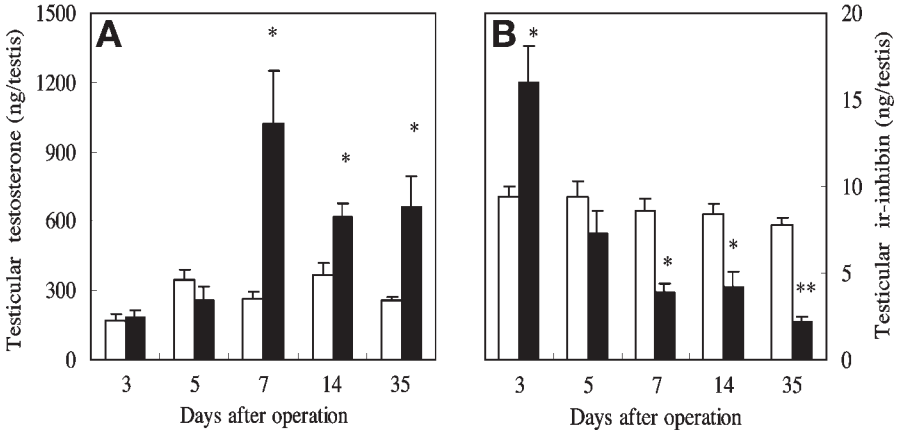

Fig. 8. Changes in testicular contents of (**A**) testosterone and (**B**) (ir-) inhibin in male rats after bilateral efferent duct ligation (solid bar) or sham-operation (open bar). Each value represents the mean ± SEM of five animals. *$p < 0.05$; **$p < 0.001$ compared with the control value.

in circulation, under the presence of elevated LH concentrations, may result from the effects of decreased blood flow through the testis *(29)*.

In conclusion, the present study demonstrated that EDL induced severe impairment of Sertoli cells and the damage of Sertoli cells resulted in disruption of spermatogenesis and inhibins secretion. On the other hand, elevated levels of LH accelerate hyperplasia of Leydig cells, although testicular testosterone do not release in circulation.

Materials and Methods

Animals and Treatments

EDL was carried out bilaterally in adult male Wistar rats (250–350 g, 3 mo of age) using the technique described by Smith *(4)*. The rats were anesthetized by ether and both testes were exteriorized through a median suprapubic incision. The vasa efferentia of testis were located by observing the oval avascular area, which marks the point at which the

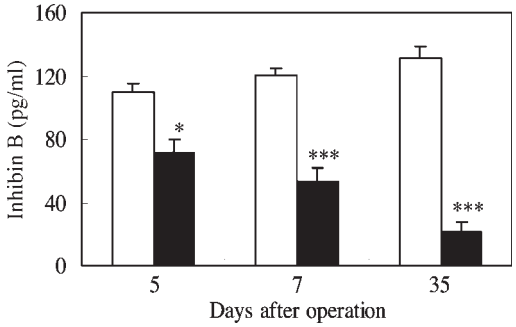

Fig. 9. Changes in plasma concentrations of inhibn B in male rats after bilateral efferent duct ligation (solid bar) or sham-operation (open bar). Each value represents the mean ± SEM of five animals. *$p < 0.05$; ***$p < 0.0001$ compared with the control value.

ductuli originate from the surface of the testis. A round suture needle and fine cotton thread were used to make two ligatures, one from the ventral and one from the dorsal aspect of the testis. The ligature was placed as close as possible to origin of the vasa efferentia, taking care not to disrupt the blood supply to the testis. Sham-operated animals served as controls. Rats were housed in a room with controlled illumination (14 h light:10 h darkness and lights on at 05:00 h) and temperature (22–25°C), with free access to commercial pellets (CE-2, Nosan Corporation, Yokohama, Japan) and given tap water *ad libitum*. All procedures were carried out in accordance with the guidelines established by Tokyo University of Agriculture and Technology.

Sample Collection

Blood samples were collected by decapitation in individual heparinized centrifuge tubes, and plasma samples were immediately obtained by centrifugation at 1700*g* for 15 min at 4°C. Plasma samples were stored at –20°C until assayed for FSH, LH, ir-inhibin, inhibin B, and testosterone. Right side testes were fixed in 4% paraformaldehyde (Sigma Chemical Co., St. Louis, MO, USA) in 0.05 *M* PBS, pH 7.4 for morphological and immunohistochemical observation. Left side testes were snap frozen for analysis of sperm count.

Sperm Concentration

The concentration of spermatozoa in epididymis was estimated in the sperm suspension of the right cauda epididymidis by the method described previously *(30)*. In brief, epididymis was homogenized in 20 mL saline by a homogenizer (Physcotron; Microtech Nition, Chiba, Japan) for 10 s, followed by sonication using a sonicater (TOMY, SEIKO Co., LTD, Tokyo, Japan) for 2 min on ice. One drop of extracted emulsion was placed on a hemocytometer after adequate dilution with saline, and sperm heads were then counted under a phase contrast microscope.

The concentration of spermatozoa in the testes was examined by the method described previously *(31)*. In brief, tunica albuginea was removed from the testes, which was then homogenized in 10 mL saline by a homogenizer (Physcotron; Microtech Nition) for 10 s, followed by sonication using a sonicater (TOMY, SEIKO Co.) for 3 min on ice. One drop of extracted emulsion was placed on a hemocytometer after adequate dilution with saline, and sperm heads were then counted under a phase contrast microscope. The remaining extracts were centrifuged at 38,000*g* for 30 min at 4°C, and supernatants were stored at –20°C for hormone assays.

Computer-Assisted Sperm Motility Analysis

Semen from the cauda epididymidis was collected into a 1.5 mL tube containing 1 mL of modified Tyrode's medium (3 μL semen sample from the cauda epididymidis diluted with 1 mL modified Tyrode's medium). The sperm motility was measured by computer-assisted sperm analysis (CASA) by using C.IMAGING C.MEN system (C.IMAGING Systems, Compix Inc, Tualatin, OR, USA). Briefly, diluted sperm suspensions were placed in prewarmed slide chambers with depths of 20 μm. The slides were viewed using Olympus microscope (Olympus BX50F, Olympus Optical Co., Tokyo, Japan) equipped with a × 4 dark field optics and a video camera (CCD XC77, Sony Co., Tokyo, Japan) connected to personal computer. The temperature of the microscope stage was maintained at 37°C throughout the observation by a stage warmer (MP-10DM, Kitazato Supply Co., Tokyo, Japan). CASA was performed using the C.IMAGING C.MEN system operating with C.IMAGING software. Our CASA system was based on the analysis of 15 consecutive, digitalized photographic images obtained from a single field. These 15 consecutive photographs were taken in a time lapse of 0.5 s. Two to three separate fields were taken for each sample. Percentage of motile spermatozoa (%), straight-line velocity (VSL, micron/s), curvilinear velocity (VCL, micron/s), linearity (ratio of the straight line distance to the actual tracked distance), deviation of the sperm head from the mean trajectory (ALH, mean micron), and the maximum amplitude of lateral head displacement (ALH, max micron) were determined.

Histological Analysis

Rats were killed by decapitation on d 3, 5, 7, 14, and 35 following the induction of EDL. The testicular tissue samples were immediately fixed in 4% paraformaldehyde (Sigma Chemical Co.) in 0.05 *M* phosphate-buffered saline (PBS), pH 7.4, and embedded in paraffin. The paraffin-embedded testes were sectioned at 6 μm thickness and placed on poly-L-lysine coated slide glasses. The sections were stained with hematoxylin-eosin (HE) for morphological observations. The seminiferous tubular diameters were measured under the microscope (Nikon, Tokyo, Japan). The diameters of 20 round tubules per animals were measured.

Immunohistochemistry

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 min. Sections were then incubated in 6% H_2O_2 in methanol at room temperature for 1 h, 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 h, to quench nonspecific staining. Then, the tissue sections were incubated at 37°C for 16–18 h with a monoclonal antibody raised in mouse against full proliferating cell nuclear antigen (PCNA) (Biomeda, Forster City, CA, USA) at a dilution of 1:200 in CTS. The 3β-hydroxysteroid dehydrogenase (3β-HSD) antibody used in the experiment was polyclonal antibody against human placental 3β-HSD raised in rabbits (kindly provided by Dr. J. I. Mason, Green Center for Reproductive Science, University of Texas, Southern Medical Center, Dallas, TX, USA). After incubation with the antibody, sections were treated with 0.25% (v/v) biotinylated goat anti-mouse (PCNA) and goat anti-rabbit (3β-HSD) secondary antibody (Elite ABC kit, Vector Laboratories, Burlingame, CA, USA) in CTS at 37°C for 30 min. The reaction products were visualized by treatment with 0.025% 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma) in 10 m*M* Tris-buffered saline containing 0.01% H_2O_2 for 1–30 min.

Radioimmunoassay (RIA) for FSH, LH, ir-Inhibin, and Testosterone

Concentrations of FSH, LH, ir-inhibin, and testosterone in the plasma were determined by each specific RIA. Iodinated preparations were rat FSH-I-7 and LH-I-7. 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 intraassay and interassay coefficients of variation were 3.4 and 5.3% for FSH and 7.2 and 11.2% for LH, respectively.

Plasma concentrations of ir-inhibin were measured as described previously *(32)*. The iodinated preparation was 32 kDa bovine inhibin and the antiserum used was rabbit antiserum against bovine inhibin (TNDH-1). Results were expressed in terms of 32 kDa bovine inhibin. The intra- and interassay coefficients of variation were 8.8 and 14.4%, respectively.

Testicular contents and plasma concentrations of testosterone were determined by an double-antibody RIA system with ^{125}I-labeled radioligands as described previously *(33)*. The antiserum against testosterone (GDN 250) was kindly provided by Dr. G. D. Niswender (Colorado State University, Fort Collins, CO., USA). The intra- and interassay coefficients of variation were 6.3 and 7.2%, respectively.

Enzyme-Linked Immunosorbent Assay of Inhibin B

Plasma concentration of inhibin B was determined using commercially available SEROTEC assay kits (Oxford, UK). Intraassay coefficient of variation was 3.4% for inhibin B.

Statistical Analysis

All data were expressed as means ± SEM of five animals. Comparisons between the ligated and control groups were made by Student's *t*-test. A value of $p < 0.05$ was considered to be statistically significant.

Acknowledgments

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