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The Stimulatory Role of Estrogen on Sperm Motility in the Male Golden Hamster (*Mesocricetus auratus*)

WANZHU JIN,*† KOJI Y. ARAI,‡ GEN WATANABE,*† AKIRA K. SUZUKI,§ SHINJI TAKAHASHI,‖ AND KAZUYOSHI TAYA*†

*From the *Department of Basic Veterinary Science, The United Graduate School of Veterinary Sciences, Gifu University, Gifu, Japan; †Laboratory of Veterinary Physiology and ‡Department of Tissue Physiology, Tokyo University of Agriculture and Technology, Tokyo, Japan; and §PM2.5/DEP Research Project, //Environmental Dioxin Project Group, National Institute for Environmental Studies, Ibaraki, Japan.*

ABSTRACT: To clarify the physiological roles of estrogens in the regulation of sperm motility in the golden hamster, two different approaches were used. In the first experiment, silastic tubes containing either low (low E_2 group) or high (high E_2 group) amount of estradiol-17β were implanted (Exp 1). In the second experiment, male golden hamsters were actively immunized against estradiol-17β (Exp 2). In Exp 1, all sperm motility parameters (including motility, straight velocity, curvilinear velocity, beat/cross frequency, and mean amplitude of lateral head displacement) were significantly increased except linear index in the high E_2 group as compared with controls at 20 days after the treatment. In the high E_2 group, plasma concentrations of luteinizing hormone (LH) significantly increased, whereas levels of circulating testosterone decreased significantly. Plasma concentrations of follicle-stimulating hormone (FSH) and immunoreactive inhibin were not affected by the treatment with estradiol-17β. In the Exp 2, titer of circulating antibodies to estradiol-17β consistently increased after the second immunization until the end of experiment (16 weeks). The sperm motility, straight velocity, and curvilinear velocity were significantly decreased after active immunization to estradiol-17β. Concentrations of circulating LH and FSH were also decreased significantly by the treatment. In conclusion, the current observations indicate that estradiol-17β affects sperm motility in adult male golden hamsters.

Key words: Estrogen immunization, gonadotropin.

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It is well established that estradiol-17β is synthesized by Leydig cells in response to luteinizing hormone (LH) and by Sertoli cells in response to follicle-stimulating hormone (FSH) in male mammals. A previous paper has indicated that germ cells are also a possible source of estradiol-17β in the male rat (Carreau, 2001). Previous studies have demonstrated that estradiol-17β is involved in the regulation of male reproductive processes through estrogen receptors (ER). Both ERα and ERβ were widely expressed in the testis and accessory sex organs throughout development and in adulthood in the male (Danzo et al, 1983; Fisher et al, 1997; Hess et al, 1997; Saunders et al, 1997, 1998). Studies in male mice, rats, and monkeys revealed the presence of ERα in the Leydig cell, rete testis, efferent duct, and epididymis, as well as in the pituitary, with some species-dependent differences (Kuiper et al, 1997). Expression of ERα was particularly intense in the efferent duct, which transports spermatozoa from the testis to the epididymis (Hess et al, 2001). In contrast, ERβ was expressed in the testis, including Sertoli cells, Leydig cells, and germ cells, as well as in the epididymis, prostate, and seminal vesicle. These results suggest that testicular estrogens directly affect testicular cells or male reproductive tracts.

Although physiological importance of estrogens in male rodents was suggested by previous studies using the rat and mouse, reproductive features of the male vary between species, even within rodents. For example, the golden hamster is a seasonal breeding species. In addition, regulation of pituitary gonadotropin by testicular feedback system in the golden hamster is likely different from that of rat (Kishi et al, 2000). Therefore, it is profitable to study male fertility in many species for understanding common and species-specific features of male reproduction. However, studies on reproductive features of the male golden hamster are quite limited. Therefore, we used this species in this study to extend our knowledge about its reproductive features.

Several previous studies were designed to examine physiological roles of estrogens in male mice and rats by

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

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using gene-targeting techniques (Eddy et al, 1996; Fisher et al, 1998; Krege et al, 1998), aromatase inhibitors (Turner et al, 2000; Gerardin and Pereira, 2002), and estrogen antagonists (Gill-Sharma et al, 1993; Balasinor et al, 2001) and have suggested physiological importance of estrogens in male fertility. However, it is difficult to evaluate physiological roles of estrogens during adulthood by knockout studies because the knockout mice have been affected by deficiency of estrogen action throughout their life. In addition, aromatase inhibitors and estrogen antagonists induce some effects besides inhibition of estrogen action. For example, administration of an aromatase inhibitor may cause accumulation of androgens because the drug inhibits conversion of androgens into estrogens. A widely used estrogen antagonist tamoxifen shows genotoxicity (Phillips, 2001) and site-specific effects (Jordan and Morrow, 1999). Therefore, in the present study, two complementary approaches were used to clarify the physiological roles of estrogen in the regulation of sperm motility; the first method is the treatment of low and high amounts of estradiol-17β (experiment 1), and the second method is immunoneutralization of endogenous estradiol-17β (experiment 2). The computer-assisted sperm analysis system was used to examine effects of estradiol-17β on sperm motility parameters.

Materials and Methods

Animals and Blood Samples

Adult male golden hamsters (*Mesocricetus auratus*) (3 months old) were used. They were kept under controlled conditions of temperature (22°C–25°C), humidity (50%–60%), and lighting (14:10 hours light to dark cycles; lights on at 0500 hours). Food and water were available ad libitum. All experimental procedures were carried out in accordance with requirements established under the Guide for the Care and Use of Laboratory Animals by Tokyo University of Agriculture and Technology.

Experiment 1: Effect of Exogenous Estradiol-17β

The aim of the first experiment was to determine whether exogenous estradiol-17β affects epididymal sperm motility. Steroid treatment procedure was according to the previous paper (Ebling et al, 2000). The male golden hamster was subcutaneously implanted with a 1.0-cm silastic tube (1.59-mm id, 3.18-mm od; Osteotec, Christchurch, United Kingdom) containing 2% crystalline estradiol-17β (Sigma Chemical Co, St Louis, Mo) and 98% crystalline cholesterol (Sigma) (low E_2 group, n = 8) or a 0.5-cm silastic tube containing crystalline estradiol-17β alone (high E_2 group, n = 10) under ether anesthesia. The control animal was implanted with a 1.0-cm tube containing crystalline cholesterol alone (n = 6). All silastic tubes were plugged at both ends with silicone medical adhesive (Osteotec). Before the implantation, the silastic tubes were washed three times in 70% ethanol, then incubated at 37°C for 24 hours in 0.01 M phosphate-buffered saline (PBS) (pH 7.4) for stabilizing release rate of estradiol-17β. Groups of animals were killed by decapitation at 20 days after the implantation. Blood samples were centrifuged at 1200 × *g* at 4°C for 15 minutes and plasma were separated and stored at −20°C until assayed for FSH, LH, immunoreactive (ir-) inhibin and testosterone. Testes, epididymides, and seminal vesicle-coagulating gland complexes (SV+CG) were collected for weighing wet weights. Epididymides were further subjected to sperm motility analysis.

Experiment 2: Active Immunization Against Endogenous Estradiol-17β

Estradiol-17β conjugated with bovine serum albumin (estriol-6-[*O*-carboxymethyl]oxime: BSA 6-ketoestriol 6-CMO: BSA) was purchased from Steraloid Inc (Newport, RI). The conjugate (1 mg) was dissolved in 1 mL of saline and the solution was mixed with an equal volume of Freund's complete adjuvant. Eight hamsters were subcutaneously injected with 200 μL of the suspension four times during the period studied. First, second, and third immunizations were performed at 2-week intervals and fourth injection was given at 4 weeks after the third injection. Control animals (n = 6) received a mixture of saline and the adjuvant. Blood samples were obtained from jugular vein just before each immunization for checking titer of antibodies to estradiol-17β. Further, plasma samples were obtained at 12 and 16 weeks after the first immunization. They were killed by decapitation at 16 weeks after the first immunization. Testes, epididymides, and SV+CG were collected for weighing wet weights. Epididymides were further subjected to sperm motility analysis.

Titer Check of Antibodies Against Estradiol-17β

Changes in titers of anti-estradiol-17β antibodies in plasma were determined by measuring the binding of ^{125}I-labeled estradiol-17β as reported previously (Kaneko et al, 1995a). The plasma was diluted 1:1000 with 0.05 M PBS (pH 7.4) containing 1% BSA, and the diluted samples were incubated with 5000 counts per minute of ^{125}I-labeled estradiol-17β at 4°C for 24 hours in a total volume of 200 μL. To separate bound radioligands, 100 μL of 1% bovine gamma globulin in PBS and 500 μL of 25% polyethylene glycol in PBS were added, and the mixture was agitated for 3 minutes. After centrifugation at 1700 × *g* at 4°C, radioactivity of the precipitate was counted in a gamma counter. Estradiol-17β binding activity was expressed as a percentage of total count added.

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

Plasma concentrations of FSH were measured as previously described (Bast and Greenwald, 1974; Kishi et al, 1995) by National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) RIA kit for rat FSH, using anti-rat FSH (S-11), NIDDK rat FSH-I-7 for iodination, and NIDDK rat FSH (RP-2) as a reference standard. Plasma concentrations of LH were measured as previously described (Bast and Greenwald, 1974; Kishi et al, 1995) by NIDDK RIA kit for rat LH, using anti-rat LH (S-10), NIDDK rat LH-I-8 for iodination, and NIDDK rat LH (RP-2) as a reference standard. Serial dilutions of female golden hamster serum pools revealed concentration curves parallel to standard FSH and LH curves in the RIAs (data not shown). 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 (Hamada et al, 1989), which was shown to be applicable to the hamster (Kishi et al, 1995). The antiserum used was raised in rabbits against bovine inhibin (TNDH-1). Purified bovine 32-kDa inhibin was used for radioiodination and standard. The assay system does not distinguish dimeric inhibin from α-subunit monomer (Kaneko et al, 1995b). The intra- and interassay coefficients of variation were 8.8% and 14.4%, respectively.

Plasma concentrations of estradiol-17β and testosterone were determined by a double-antibody RIA system using ^{125}I-labeled radioligand, as described previously (Taya et al, 1985). Antiserum against estradiol-17β (GDN #244) (Korenman et al, 1974) and testosterone (GDN #250) (Gay and Kerlan, 1978) were kindly supplied by Dr G. D. Niswender (Animal Reproduction and Biotechnology Laboratory, Colorado State University, Fort Collins, Colo). The intra- and interassay coefficients of variation were 5.8% and 11.4% for estradiol-17β, and 6.3% and 7.2% for testosterone, respectively.

It should be noted that because the assays for FSH, LH, and inhibin are a heterologous system, values of these hormones are rational.

Computer-Assisted Sperm Motility Analysis

The sperm motility parameters were obtained using C. IMAGING computer-assisted sperm motion analysis system (Compix Inc, Cranberry Township, Penn). Sperm in one drop of caudal epididymal fluid were incubated at 37°C for 3 minutes in medium 199 (Biocell, Cal) containing 2.5 mM HEPES (pH 7.2) and 0.5% BSA (Sigma). After the incubation, an aliquot of this solution was diluted 10- to 20-fold with the medium, and 10 μL was placed into the microcell-HAC chamber, which has a depth of 50 μm (Conception Technologies, San Diego, Cal). Analyses of motile 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, Japan), was used by 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 mean amplitude of lateral head displacement (ALH) = 3.5; pixel scale 3.26 μm/pixel; maximum average number of cells/field = 30; cell size range = 350–1600 pixel. The following characteristics were analyzed: the percentage of motile spermatozoa, curvilinear velocity (the total distance traveled divided by the total time the cell was tracked), straight velocity (straight line distance), ALH (deviation of the sperm head from the mean trajectory), beat/cross frequency, linearity (ratio of the straight line distance to the actual tracked distance), and the percentage of circular cells.

Statistics

One-way analysis of variance was performed. Significance between two means was evaluated with Student's *t* test, and significance among more than two means was determined by Duncan's multiple range test (Steel and Torrie, 1960). All data are presented as means ± SEM. Differences were considered significant when $P < .05$.

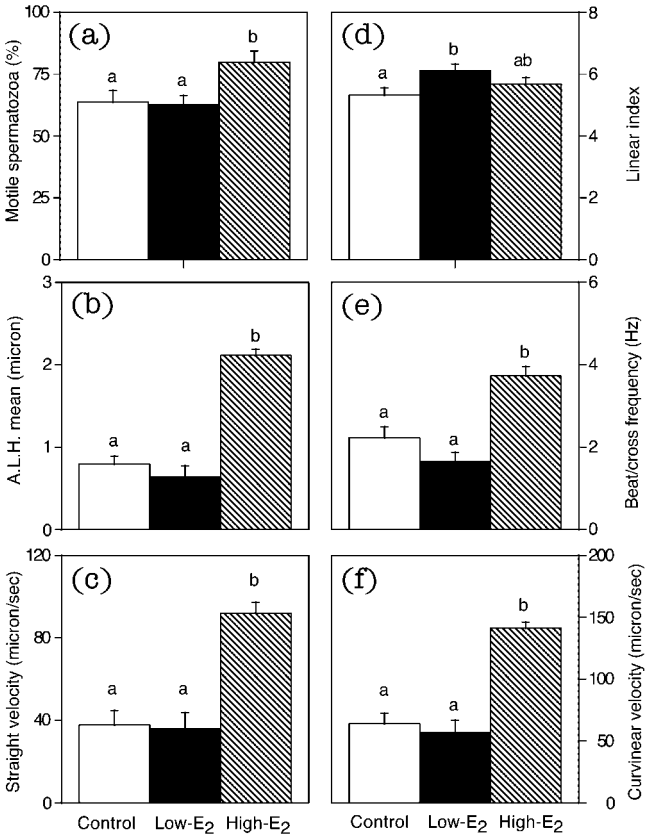

Figure 1. Effects of long-term treatment with estradiol-17β on sperm motility parameters. Male hamsters were implanted with silastic capsules containing either low or high amounts of estradiol-17β. Controls received implants containing cholesterol alone (CHO). Hamsters were killed at 20 days after the treatments. Values are means ± SEM for 6–10 hamsters. Bars with different superscripts were significantly different ($P < .05$).

Results

Experiment 1: Effects of Exogenous Estradiol-17β

Sperm Motility Parameters (Figure 1)—The percentage of motile spermatozoa was significantly high in the high E_2 group as compared with the control group treated with cholesterol alone (Figure 1a). In the high E_2 group, ALH mean (Figure 1b) and straight velocity (Figure 1c), beat/cross frequency (Figure 1e), and curvilinear velocity (Figure 1f) were significantly high as compared with the control group. On the other hand, in the low E2 group, only linear index (Figure 1 day) was significantly higher than the control group.

Plasma Concentrations of FSH, LH, Estradiol-17β, ir-Inhibin, and Testosterone (Figure 2)—Plasma concentrations of estradiol-17β were increased in a dose-dependent manner (Figure 2c). Plasma concentrations of LH showed a dose-dependent increase in response to the treatment

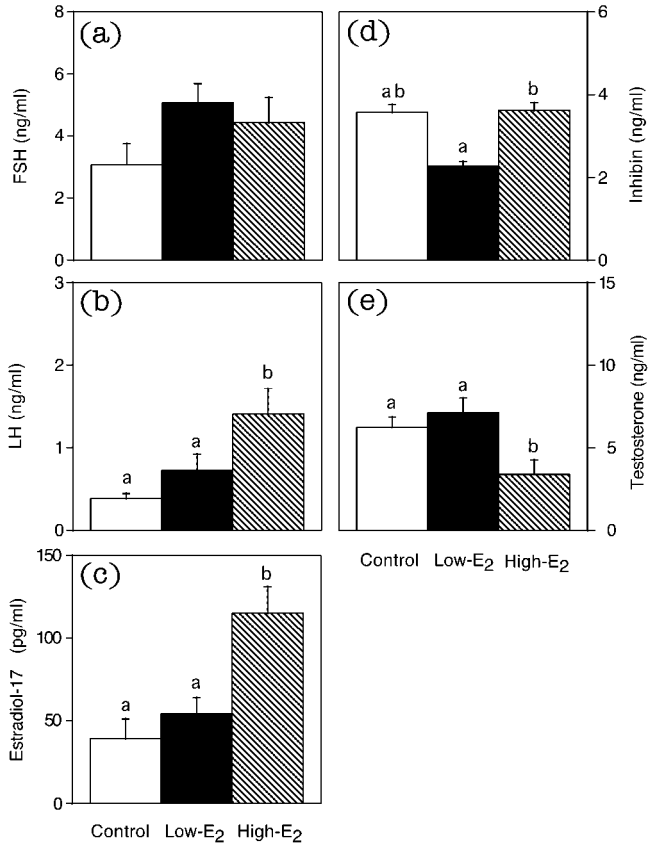

Figure 2. Plasma concentrations of gonadotropins and gonadal hormones after implantation of silastic capsules containing either low (Low E_2) or high (High E_2) amounts of estradiol-17β. Control animals received implants containing cholesterol alone (CHO). Hamsters were killed at 20 days after the treatments. Values are means ± SEM for 6–10 hamsters. Bars with different superscripts were significantly different ($P < .05$).

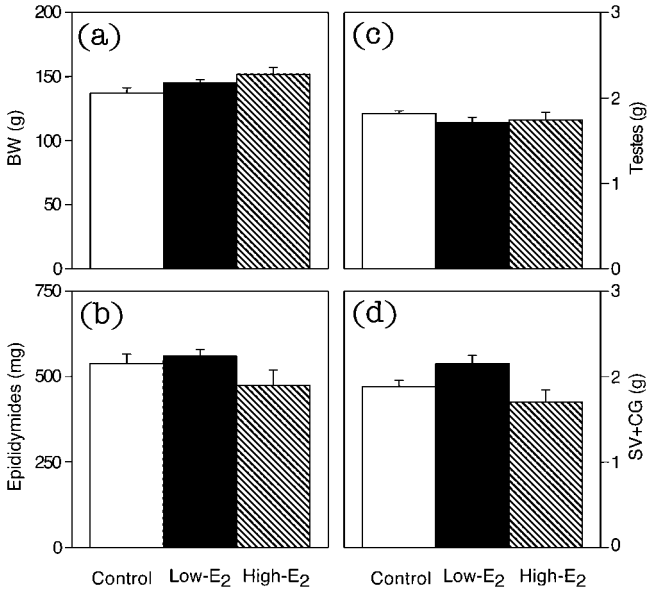

Figure 3. Effects of long-term treatment with estradiol-17β on weights of bodies (BW), testes, epididymides (EP), and seminal vesicles with coagulating glands (SV+CG) in male golden hamsters. The hamsters were implanted subcutaneously (s.c.) with silastic capsules containing either low (Low E_2) or high (High E_2) amounts of estradiol-17β. The control group received s.c. implants containing cholesterol alone (CHO). They were killed at 20 days after the implantation. Each value represents mean ± SEM for 6–10 hamsters. Bars with different superscripts are significantly different ($P < .05$, Dancan's multiple range test).

with estradiol-17β, and the level is significantly high in the high E_2 group as compared with the control group (Figure 2b). Plasma concentrations of testosterone were significantly low in the high E_2 group as compared with the control group (Figure 2e). Plasma concentrations of FSH and ir-inhibin were not affected by administration of estradiol-17β (Figure 2a and d).

Weights of Reproductive Organs (Figure 3)—Treatment with either low or high dose of estradiol did not affect weights of bodies, epididymides, testes, and SV+CG.

Experiment 2: Active Immunization Against Endogenous Estradiol-17β

Anti-Estradiol-17β Titer in Plasma (Figure 4)—The time course of antibody development in the immunized animals as determined by binding with the ^{125}I-labeled estradiol-17β was shown in Figure 4. Antibodies against ^{125}I-labeled estradiol-17β were produced by all hamsters that received the antigen. The anti-E_2 titers rose after the sec-

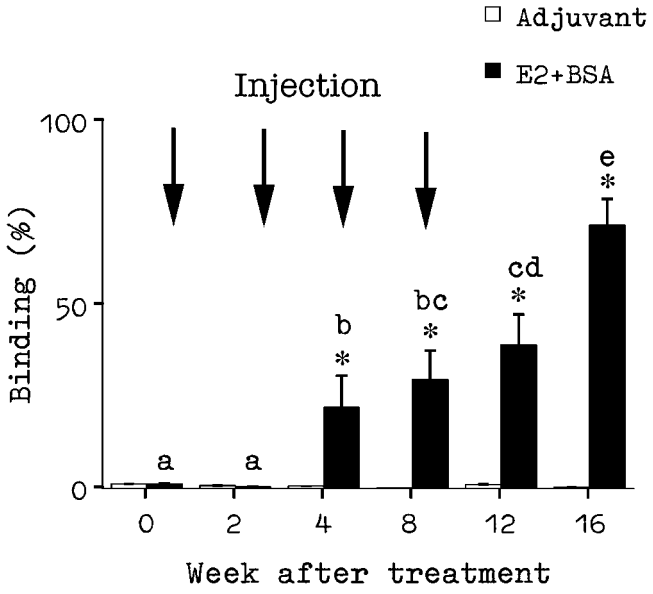

Figure 4. Changes in titers of anti-estradiol-17β antibodies after active immunization. Values are means ± SEM for six (control) or eight (immunized group) hamsters. Animals were immunized at 0, 2, 4, and 8 weeks (arrows). *, significant as compared with control ($P < .05$). Bars with different superscripts were significantly different ($P < .05$).

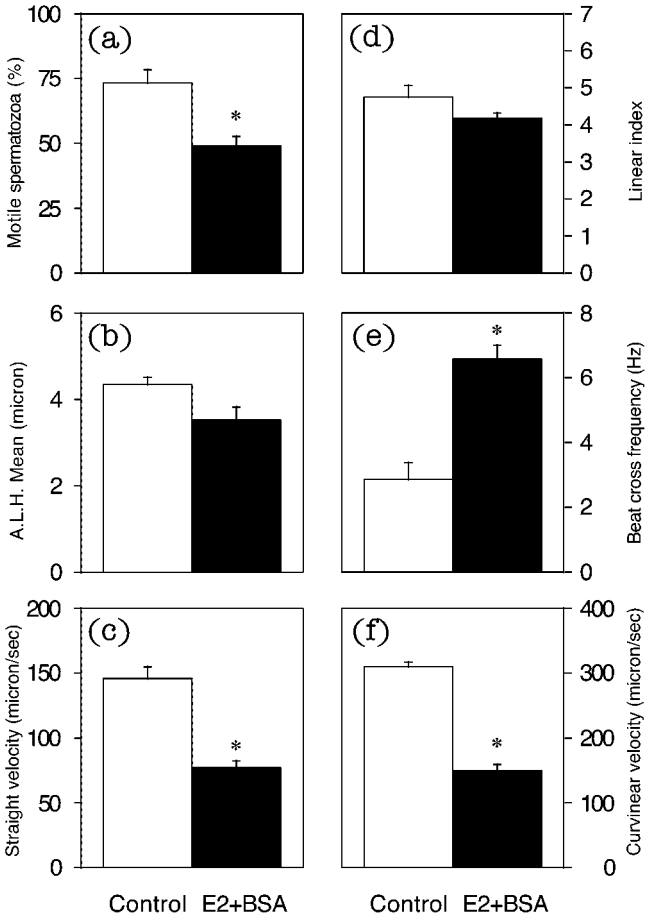

Figure 5. Changes in sperm motility parameters after active immunization against estradiol-17β. Values are means ± SEM for six (control) or eight (immunized group) hamsters. *, significant as compared with control ($P < .05$).

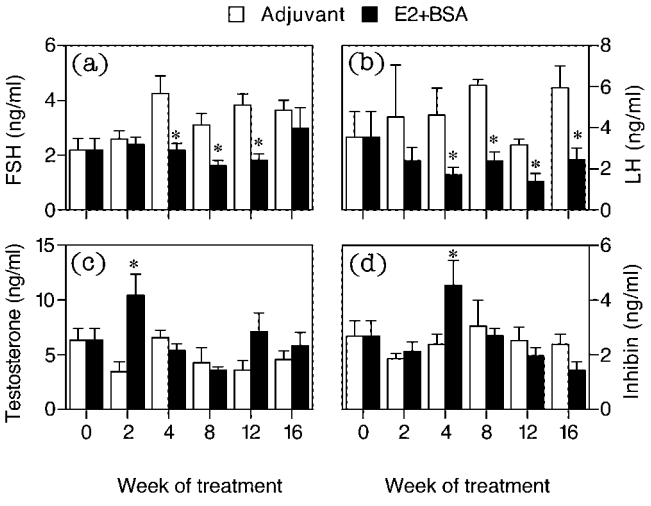

Figure 6. Changes in reproductive hormones after active immunization against estradiol-17β. Animals were immunized at 0, 2, 4, and 8 weeks. Values are means ± SEM for six (control) or eight (immunized group) hamsters. *, significant as compared with control ($P < .05$).

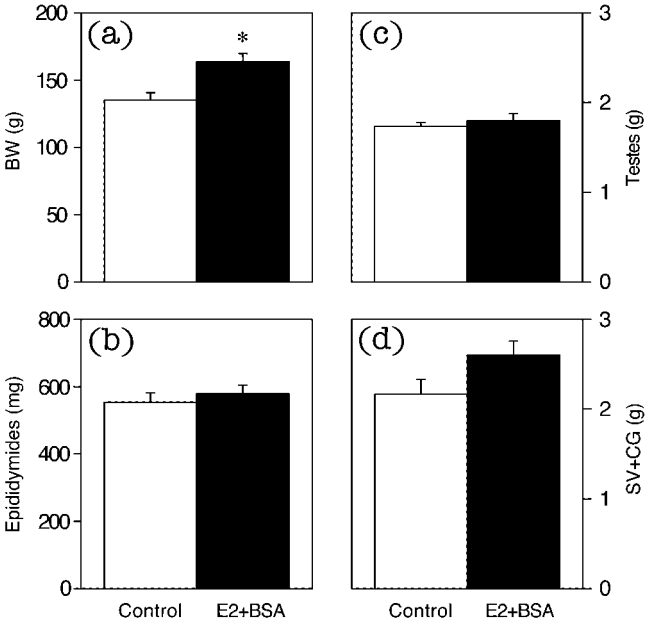

Figure 7. Effects of active immunization against estradiol-17β on weights of bodies and reproductive organs. Animals were immunized at 0, 2, 4, and 8 weeks and killed at 16 weeks. Values are means ± SEM for six (control) or eight (immunized group) hamsters. *, significant as compared with control ($P < .05$).

ond immunization, and it reached 72% at 16 weeks after the primary immunization.

Sperm Motility Parameters (Figure 5)—The percentage of motile sperm (Figure 5a), straight velocity (Figure 5c), and curvilinear velocity (Figure 5f) of spermatozoa were significantly low in the immunized group as compared with the control group. ALH mean (Figure 5b) and linear index (Figure 5 d) tended to be low in the immunized group as compared with the control group, whereas there is no significant difference between these groups.

Plasma Concentrations of FSH, LH, Testosterone, and ir-Inhibin (Figure 6)—Plasma concentrations of FSH were significantly lower in the immunized group than in the control group from 4 to 12 weeks after the primary immunization (Figure 6a). Plasma concentrations of LH were also significantly lower in the immunized group than in the control group from 4 to 16 weeks (Figure 6b). Plasma concentrations of testosterone and ir-inhibin did not differ significantly between the two groups except at 2 weeks for testosterone and 4 weeks for ir-inhibin (Figure 6c and d).

Weights of Reproductive Organs (Figure 7)—Body weights were significantly high in the immunized group as compared with the control group (Figure 7a). There are no significant differences between the immunized and control groups in weights of testes, epididymides, and SV+CG, respectively (Figure 7b through d).

Discussion

The present results clearly demonstrated that estradiol-17β stimulates sperm motility of the adult male golden hamster. Results of the first experiment showed that all sperm motility parameters except linear index were increased at 20 days after treatment with the high dose of estradiol-17β. In addition, in the second experiment, the sperm motility, straight velocity, and curvilinear velocity decreased by the active immunization against estradiol-17β. These results clearly indicate that estradiol-17β treatment severely affects not only the ability of sperm to move in a forward direction but also their vigor. Previous data demonstrated that administration of estradiol benzoate increased the proliferation of type A spermatogonia when delivered to infant rats from 5 to 11 days of age (Lindgren et al, 1976). This study is supported by several following papers, which showed stimulatory effects of estradiol-17β on spermatogenesis. Kula (1988) demonstrated a stimulatory role of estradiol-17β in spermatogenesis in the rat. Full germ cell development can be induced by estradiol-17β in the hypogonadal mice (HPG) (Ebling et al, 2000). Neonatal exposure of rats to low levels of estrogens can advance the first wave of spermatogenesis at puberty, although it is unclear whether this is due to direct effects of the estrogen or whether it is due to an elevation of FSH levels associated with the treatment with estrogens (Atanassova et al, 2000). However, the doses of estradiol-17β used in the present study are far lower than the 15 μg per day estradiol benzoate treatment in the previous study (Lindgren et al, 1976). Therefore, it is not likely that the doses of estradiol-17β used in this study affect spermatogenesis in the hamster. Pentikainen et al (2000) reported that low concentrations of estradiol-17β effectively inhibited male germ cell apoptosis, suggesting that estrogens act as a germ cell survival factor in men. In contrast, a few studies reported effects of estradiol-17β on sperm motility. Robaire et al (1987) reported that the percentage of motile sperm was significantly reduced in rats treated with ethinyl estradiol after 1 week of treatment at 10 mg/kg body weight and after 2 weeks of treatment at 1 mg/kg body weight. Goyal et al (2001) also reported that the treatment with supraphysiological levels of estradiol-17β caused significant reduction in epididymal sperm numbers and sperm motion in the adult male rat. However, those authors used a very high dose of estradiol-17β compared with the present study. The supraphysiological dose in the previous study might cause toxicity for the reproductive systems and negative effects on sperms. Studies of ERα knockout (ERKO) mice suggested that estrogens are important for sperm production (Eddy et al, 1996) and fluid reabsorption within the efferent ducts (Hess et al, 1997). Targeted disruption of ERα resulted in damage to spermatogenesis and subsequent infertility (Eddy et al, 1996). The ERgb knockout mice showed hyperplasia of the bladder and prostate epithelium in older males. However, the reproductive tract appears grossly normal in two types of ERKO mice (Krege et al, 1998). On the other hand, aromatase knockout mice have generated phenotypes implicating a role of estrogen in determining male fertility (Fisher et al, 1998). Previous papers reported that exposure of the neonatal/fetal male rodent to exogenous estrogen can cause a range of abnormalities of the reproductive system, including testes atrophy and abnormalities of the rete testis (Arai et al, 1983; Gaytan et al, 1986). It has generally been concluded that the indirect action is responsible for the adverse effects of neonatal estrogen exposure on the testis (Arai et al, 1983; Bellido et al, 1990). Because the maximal treatment period was 20 days in the present study, only late spermatids and sperms that were already within the epididymis would be examined for sperm motility. Therefore, the effects of the treatments on sperm motility might be due to effects on the epididymis.

The hormone profiles in the first experiment of the present study showed that plasma LH levels significantly increased at 20 days after treatment with the high dose of estradiol-17β. In addition, results of the second experiment clearly showed that plasma concentration of FSH and LH declined after the active immunization of estradiol-17β. These results corroborate previous findings, which showed a decrease in circulating LH by a low dose of anti-estrogen tamoxifen in the adult rat (Gill-Sharma et al, 1993). Although treatment with estradiol-17β decreased serum FSH concentrations in wild-type mice (as expected), it induced a small (but significant) rise in circulating FSH levels in male HPG mice (Ebling et al, 2000) and in the neonatal rat (Atanassova et al, 2000). The reason for the discrepancy is not known yet. With respect to testosterone, plasma levels of testosterone declined significantly at 20 days after treatment with high levels of estradiol-17β, although plasma levels of LH increased. Similarly, active immunization against estradiol-17β temporarily increased circulating testosterone. These results suggest that estradiol-17β may directly suppress testicular secretion of testosterone. At present, it is not known whether estradiol-17β directly affected sperm motility through ERs in spermatozoa. It is also possible that the changes in plasma concentrations of LH and FSH affected sperm motility, although testosterone secretion did not reflect the changes in plasma gonadotropins.

In conclusion, the present study clearly demonstrated that estradiol-17β has stimulatory effects on sperm motility and secretion of gonadotropin in the golden hamster, and has a suppressive effect on testosterone secretion. Further studies are required to reveal mechanisms responsible for these responses.

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