Effects of anabolic steroid (19-nortestosterone) on the secretion of testicular hormones in the stallion

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The aim of this study was to clarify the effect of anabolic steroids on the testicular endocrine function of mature stallions. Mature thoroughbred stallions were treated with 800 mg nandrolone decanoate every 3 weeks for 3 months. After the first treatment, plasma concentrations of LH, immunoreactive inhibin and testosterone decreased rapidly to the nadir. These hormones were maintained at significantly lower concentrations compared with concentrations in intact stallions. Histology of the testicular tissue indicated the arrest of advanced spermatogenesis in the seminiferous tubules and a severe depletion of the number of Leydig cells in the interstitial compartment as a result of treatment. Most of the immunopositive cells for the inhibin α-subunit and steroidogenic enzymes in the interstitial compartment decreased below detectable amounts, whereas immunopositive reactions of inhibin α-subunit in the seminiferous tubules were clearly observed. In conclusion, the treatment of mature stallions with nandrolone decanoate caused a decrease in the secretion of ir-inhibin and testosterone from the testis, the depletion of the number of Leydig cells and a decrease below detectable amounts of inhibin α-subunit and steroidogenesis enzymes. The concentration of ir-inhibin in the peripheral blood may be a useful marker for the examination of testicular activity in stallions being treated with anabolic steroids.

Introduction

Anabolic steroids are synthetic derivatives of testosterone, the effects of which are characterized by increased anabolic activity and decreased androgenic activity. One of the most traditional and also popular anabolic steroids for horses is 19-nortestosterone. Anabolic steroids have been used widely for therapy, growth acceleration and performance improvement in the horse industry, but long-term treatment or large doses have serious side effects on reproductive function.

Anabolic steroids have adverse effects upon the reproductive function of stallions. Administration of anabolic steroids showed adverse effects on testicular function, decreasing testis mass, scrotal width, daily sperm production and the numbers of Leydig cells (Squires et al., 1982; Garcia et al., 1987; Jackson et al., 1991; Koskinen 1991). These detrimental effects are thought to result from the inhibition of the secretion of gonadotrophins from the pituitary gland (Squires et al., 1982; Turner and Irvine 1982) which, in turn, results in the suppression of the secretion of testicular hormones from the testis. Little information is available on the effects of anabolic steroids on the hormone regulation of peripheral concentrations of gonadal hormones, such as testosterone and oestradiol, in mature stallions, despite the fact that these are strong indicators of the condition of reproductive function (Berndtson et al., 1974; Nagata et al., 1998a,b).

Stallion testes secrete a large amount of immunoreactive (ir)-inhibin in circulation, and the amount of circulating ir-inhibin shows a clear annual change in relation to the reproductive season (Roser et al., 1994; Nagata et al., 1998a,b), indicating that the circulating concentrations of inhibin reflect testicular functions. Furthermore, we have described the cellular localization of inhibin subunits and two key steroidogenic enzymes, 3β-hydroxy-dehydrogenase (3β-HSD) and cytochrome P₄₅₀ aromatase (aromatase), and discussed the secretion sites of testicular hormones in the stallion testis (Nagata et al., 1998b). Anabolic steroids may influence the localization sites of inhibin and steroidogenic enzymes in the testis.

In the present study, 19-nortestosterone compound was injected into mature stallions for 3 months and the changes in circulating concentrations of ir-inhibin, testosterone, FSH and LH were analysed to investigate the effect of anabolic steroids on the testicular function of stallions. In addition, the immunolocalization sites of the inhibin α-subunit and two steroidogenesis enzymes, 3β-HSD and aromatase, in the testes were determined after treatment with anabolic steroid.
Materials and Methods

Animals and treatment

Between June and September, four healthy mature thoroughbred stallions were treated once every 3 weeks for 3 months with an i.m injection in the neck region of 800 mg nandrolone decanone, an ester of 19-nortestosterone, in sesame oil (Fuji Pharm., Tokyo). Five healthy mature stallions were used as intact controls, receiving injections of sesame oil only.

Blood samples

All blood samples (10 ml) were taken by venepuncture from the jugular vein of each horse at 09:00 h. The blood sampling was performed 1, 3, 5 and 7 days after the injection for 1 week and then once per week for 4 weeks after the first injection, and followed continuous sampling every 2 weeks until 4 weeks after the last treatment. All blood samples were immediately centrifuged at 2000 g for 10 min at 4°C and individual plasma samples were stored at −20°C until assayed for LH, FSH, ir-inhibin and testosterone.

Testicular samples

Three treated stallions and all but one control stallion (which had been moved to another farm) were castrated under general anaesthesia (10% (v/v) glycerol guaiacolate ether) after the last collection of blood samples. Testes were weighed, and sections of the testicular parenchyma (0.5–1.0 cm³) were taken from the centre of the testes. These sections were immediately fixed in 4% (w/v) paraformaldehyde (Sigma Chemical Co., St Louis, MO) in 0.01 mol PBS 1×, pH 7.4, and embedded in paraffin wax. The paraffin-embedded testes were serially sectioned at 6 μm thickness and placed on silane-coated slides (Dako Japan Co. Ltd, Kyoto).

Immunohistochemistry of inhibin α-subunits, 3β-HSD and aromatase

After the sections of testicular tissue were deparaffinized with xylene, they were subjected to antigen retrieval by autoclaving in 0.01 mol sodium citrate buffer 1×, pH 6.0 at 121°C for 15 min. The sections were then incubated in 3% (v/v) H₂O₂ in methanol at room temperature for 30 min and then in 0.5% (w/v) casein–Tris saline (0.05 mol Tris–HCl 1× with 0.15 mol NaCl 1×, pH 7.6) (CTS) at 37°C for 1 h to quench nonspecific staining. A section was incubated for 8–12 h at 4°C with polyclonal antibody against the inhibin α-subunit at a dilution of 1:2000–4000 in CTS. The antibody of inhibin α-subunit made use of a rabbit antisera against a synthetic [Tyr30]α-chain (1–30) NH₂ fragment of pig inhibin (Ueno et al., 1987), kindly provided by N. Ling (Neuroendocrine, Inc., San Diego, CA) and immunoreaction of this antibody to inhibin in the stallion testis was confirmed by Nagata et al. (1998a). After this incubation, the sections were treated with 0.5% (v/v) biotinylated goat anti-rabbit secondary antibody (ABC kit Elite, Vector Lab, Inc., Burlingame, CA) in CTS for 1 h and were incubated subsequently with 2% (v/v) avidin–biotin complex solution (ABC kit Elite, Vector Lab, Inc.) in CTS for 30 min at 37°C. The antibody bound to the sections was visualized by treating with 0.05% (w/v) 3,3′-diaminobenzidine tetra-chloride (Sigma Chemical Co.) in 10 mmol Tris-buffered saline 1× containing 0.01% (v/v) H₂O₂ for 3 min.

The serial sections were also stained with antisera against 3β-HSD and aromatase in the same manner as described for inhibin. The 3β-HSD antisera used was polyclonal antisera against human placental 3β-HSD raised in a rabbit (diluted 1:1000; kindly provided by J. I. Mason, Cecil H. and Ida Green Center for Reproductive Science, University of Texas, Southern Medical Center, Dallas, TX). The aromatase antisera used was polyclonal antisera against human placental P₄₅₀arom (R-8-1, diluted 1:2000) raised in a rabbit (kindly provided by Y. Osawa, Medical Foundation of Buffalo, Buffalo, NY). These antisera have been used previously in horse tissue (Mason et al., 1993; Eisenhuer et al., 1994).

A serial section of each testis sample was also stained with haematoxylin and eosin to investigate the structure of testis tissue.

Radioimmunoassay of immunoreactive inhibin, FSH and LH

Plasma concentrations of ir-inhibin were measured by radioimmunoassay using a rabbit antisera against purified bovine inhibin (TNDH 1) and 125I-labelled 32 kDa bovine inhibin, as described by Hamada et al. (1989) and Nagata et al. (1998a). The results were expressed in terms of amount of 32 kDa bovine inhibin. The sensitivity of the assay was 7.8 pg per tube. The intra- and interassay coefficients of variations were 8.0 and 16.2%, respectively.

Plasma concentrations of FSH and LH in all blood samples were measured by radioimmunoassay using a rabbit antisera against human FSH (no. 6; kindly provided by NIDDK NIH, Bethesda, MD) and using a rabbit antisera against ovine LH (YM no. 18; kindly provided by Y. Mori, Laboratory of Veterinary Ethology, University of Tokyo, Tokyo). Highly purified equine FSH and equine LH (both kindly provided by H. Papkoff, Hormone Research Laboratory, University of California, San Francisco, CA) were used as the standards and for iodination. The intra- and interassay coefficients of variation were 9.2 and 13.2% for FSH and 8.8 and 13.0% for LH, respectively.

Radioimmunoassay of testosterone

Testosterone in plasma samples was separated from 19-nortestosterone (the active agent of nandrolone decanoate) by HPLC before being measured by radioimmunoassay, because the antisera against testosterone showed a substantial crossreaction with 19-nortestosterone.

The HPLC equipment using a variable-wavelength UV
with nortestosterone was effluent mobile under solution H was (0.05 MA). (25-6400 Chromatographie de canoate. value significant expressed individual as (D). The immunoreactive (ir)-inhibin (●), testosterone (○), and LH (□) in mature thoroughbred stallions. Each value is expressed as the mean ± SEM of four animals. Asterisks show significant differences (*P < 0.05) compared with the pretreatment value (day 0). Arrowheads indicate injections of 800 mg nandrolone decanoate.

detector was purchased from Hitachi Co., (Tokyo). Chromatographic separations were obtained with a Cosmosil 5C_{18} packed column (4.6 mm × 150 mm inner diameter; Nacalai Tesque, Inc., Kyoto). The mobile phase was prepared by mixing acetonitrile and 0.01 mol acetic acid 1 solution (4:6). Various doses of testosterone standard solution were prepared in charcoal-treated gelidung serum (25–6400 pg ml^{-1}). Each standard solution (1 ml) and individual unknown plasma samples were inserted in SepPak tC_{18} cartridges (Waters Associates, Inc., Milford, MA). The cartridge was washed with 9 ml water and 6 ml n-hexane, and then testosterone in the cartridge was eluted with 6 ml diethyl ether. The diethyl ether elution was dried under N_{2} at 40°C. The residue was dissolved in 500 µl HPLC mobile phase and 50 µl was injected into the HPLC.

The following HPLC conditions were used: the flow rate was 1.0 ml min^{-1}; the column temperature was 40°C and detection was at 245 nm. The retention time of 19-nortestosterone and testosterone was determined in a run with each standard (8.36 min and 10.18 min, respectively). This profile was checked before every experiment. The effluent testosterone fractions from the standard solutions and the plasma samples were collected using the determined retention times, and the fraction solutions were dried under N_{2} at 60°C. The residue was redissolved in 100 µl BSA buffer (0.05 mol PBS 1 containing 1% BSA, pH 7.4).

The testosterone was determined by double-antibody radioimmunoassay systems using ^{125}I-labelled radioligands, as described by Taya et al. (1985). Antiserum against testosterone (GDN 250; Gay and Kerlen, 1978), kindly provided by G. D. Niswender (Animal Reproduction and Biotechnology Laboratory, Colorado State University, Fort Collins, CO) was used. The recovery rate of testosterone from equine plasma by this procedure was 58.6%. The intra- and interassay coefficients of variation were 6.3 and 7.2%, respectively.

**Statistical analysis**

All data are presented as means ± SEM. When there was heterogeneity of variance and the standard deviations were proportional to the means, logarithmic transformation was carried out before analysis of variance. Repeated measures analysis of variance (ANOVA) with post hoc comparison using the Newman–Keuls test was used to test for significant differences from pretreatment concentrations. The Mann–Whitney U test was used to compare the evaluations of hormone concentrations between intact and treated stallions. Pearson’s correlation coefficient was used to examine the relationship between the concentrations of each hormone. All differences with values of *P < 0.05* were considered significant.

**Results**

**Circulating concentrations of immunoreactive inhibin, testosterone, FSH and LH**

The changes in concentrations of ir-inhibin, testosterone, FSH and LH in plasma resulting from treatments with nandrolone decanoate are presented (Fig. 1). The results of ANOVA showed that the treatment with nandrolone decanoate significantly affected the concentration of ir-inhibin, testosterone and LH (*P < 0.05*). The concentrations of ir-inhibin consistently decreased after the first treatment with nandrolone decanoate and reached a significant low concentration 5 days after the treatment in comparison with the pretreatment concentration. Concentrations of testosterone and LH in plasma abruptly decreased after the first treatment within 1 day. Thereafter, the circulating concentrations of these hormones were maintained at low concentrations during the period of study. Plasma concentrations of FSH also tended to decrease owing to the treatment with nandrolone decanoate, whereas they were not significantly different compared with the pretreatment concentration. The concentrations of ir-inhibin and testosterone exhibited higher positive correlation with LH (*r* = 0.60 for ir-inhibin and *r* = 0.91 for testosterone) than with FSH (*r* = 0.33 for ir-inhibin and *r* = 0.40 for testosterone).

The plasma concentrations of these hormones during the period of the studies were compared between the nandrolone decanoate treated stallions and the intact stallions (Fig. 2). Before the treatment of nandrolone decanoate (in June), the plasma concentrations of all hormones were not significantly different between intact
and experimental stallions. After the treatment with nandrolone decanoate, the plasma concentrations of ir-inhibin, testosterone and LH were significantly lower in treated stallions than they were in intact stallions in the same month.

**Histology of testis**

The haematoxylin- and eosin-stained sections of intact and treated testicular tissues are shown (Fig. 3a,b). The intact testicular tissue had many seminiferous tubules, including a large number of spermatozoa, germ cells and Sertoli cells, and these were surrounded by interstitial tissue filled with Leydig cells with a spherical nucleus and an eosinophilic cytoplasm (Fig. 3a). The seminiferous tubules in the testes of treated animals were smaller than in those of intact controls, and there appeared to be few germinal cells and no spermatids in the seminiferous tubules of the treated stallions (Fig. 3b). In the interstitial compartment of treated stallions, there appeared to be severe depletion of the number of Leydig cells, and most of the Leydig cells showed advanced atrophy.

Immunostaining sections with antibody to the inhibin α-
subunit, 3β-HSD and aromatase of the intact stallion testes and nandrolone decanoate-treated stallion testes are shown (Fig. 3c–h). In intact stallions, the immunohistochemical localization of the inhibin α-subunit was in the Sertoli cells within the seminiferous tubules and in the Leydig cells within the interstitial compartment (Fig. 3c), whereas 3β-HSD and aromatase were observed only in the Leydig cells (Fig. 3e,g). However, in treated stallions, inhibin α-subunit positive cells were clearly observed in the seminiferous tubules, and there were only a few small positive cells in the interstitial compartment (Fig. 3d) and few 3β-HSD- and aromatase-positive cells were observed in the interstitial compartment (Fig. 3f,h).

Discussion

The concentration of testicular hormones in peripheral blood is one of the most important factors in the evaluation of testicular function after treatment with anabolic steroids. In humans, the HPLC purification step for testosterone before radioimmunoassay has been used in the investigation of male fertility with a high dose treatment of nandrolone esters (Belkien et al., 1985). The plasma concentrations of testosterone after treatments of nandrolone decanoate were also measured correctly in the present study by radioimmunoassay in combination with HPLC, although this procedure is troublesome. Circulating ir-inhibin concentration has been used as a marker of gonadal activity in sheep and monkeys (Lincoln and McNeilly, 1989; Matsubayashi et al., 1991) and can be measured directly even in animals undergoing nandrolone decanoate treatment. In the present study, the plasma concentration of ir-inhibin in stallions decreased after the first treatment with nandrolone decanoate. This is the first demonstration to our knowledge of the effect of the anabolic steroid on inhibin secretion. These results indicate that ir-inhibin in plasma is a useful indicator of testicular function in nandrolone decanoate-treated stallions.

Changes in the concentrations of ir-inhibin after the first injection of nandrolone decanoate showed a close correlation with the plasma concentration of LH rather than that of FSH. Inhibin α-subunit is localized in Leydig cells as well as Sertoli cells in the stallion testis (Nagata et al., 1998a,b) and the Leydig cells of the stallion have receptors for LH and hCG (Evans et al., 1982). Consequently, a decrease in the concentration of plasma ir-inhibin after the first injection of nandrolone decanoate must be caused by a decrease in LH concentration.

In humans and rats, treatment with anabolic steroids suppresses secretion of FSH as well as LH concentrations (Bijlsma et al., 1982; Grokett et al., 1992). In the present study, the plasma concentration of FSH decreased slightly after the first treatment with nandrolone decanoate, but the ranges during the treatment were within the normal concentration ranges observed in intact adult stallions. As inhibin has a suppressing action to secretion of FSH from pituitary glands (Ying, 1988), decreased circulating inhibin concentrations may prevent the decrease in the plasma concentration of FSH that results from treatment with nandrolone decanoate in the stallions.

The atrophic state of the Leydig cells in the testis of anabolic steroid-treated stallions indicates a reduction of the normal LH stimulation. It is possible that nandrolone decanoate acted directly on the Leydig cells in the same way as a competitive inhibitor of testosterone (Purvis et al., 1979). Stallion testes have been reported to secrete large amounts of androgens and oestrogens in relation to spermatogenesis, and 3β-HSD and aromatase are key enzymes in a pathway for synthesis of their steroid hormones in Leydig cells. In the present study, the severe depletion of Leydig cells after treatment with nandrolone decanoate caused the concentration of their steroidogenic enzymes in the testis to decrease below detectable amounts. The decrease of concentrations of 3β-HSD and aromatase below detectable amounts induces a deficiency in androgens and oestrogens, and must have a detrimental influence on spermatogenesis in the testis. After treatment with nandrolone decanoate, there was a marked decrease in the number of inhibin α-subunit-positive cells in the interstitial compartment, which must be caused by the marked decrease in plasma concentrations of ir-inhibin in the treated stallions. However, immunopositive reactions of the inhibin α-subunit within the seminiferous tubules were more marked in treated than in the intact stallions. The function of Sertoli cells appeared to be maintained throughout the study, and Sertoli cells maintain a range of plasma ir-inhibin in treated stallions that is higher than that in castrated stallions. Inhibin secretion from Sertoli cells in male animals is stimulated by FSH (Au et al., 1984). In the present study, the positive reaction of the inhibin α-subunit within Sertoli cells in the treated stallions may be due to the basal concentration of circulating FSH. However, significantly lower concentrations of plasma ir-inhibin coincident with the disappearance of Leydig cells indicate the secretion of a large amount of ir-inhibin from Leydig cells in normal stallions.

In conclusion, it is clear that anabolic steroids have a suppressing effect on the secretion of testicular hormones. This effect is likely to be caused mainly by a decrease in the circulating concentrations of LH, but not of FSH. Furthermore, it is essential that the long-term effects of the administration of anabolic steroids cause severe depletion of the number of Leydig cells and inhibition of spermatogenesis in the testes. Steroidogenesis and inhibin α-subunit production within Leydig cells also ceased, whereas inhibin α-subunit within Sertoli cells was observed clearly. These results indicate that the main source of circulating inhibin in stallions is the Leydig cells. The present study also indicates that treatment with anabolic steroid in mature stallions has highly detrimental effects to testicular function, and that the concentration of ir-inhibin in peripheral blood is a useful marker of testicular activity under treatment with anabolic steroids in stallions.

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