Concentrations of oestrone sulphate, androgens and LH in the peripheral plasma of mating stallions

G. Bono, D. Minori,* R. Gaiani and M. Mattioli

*Istituto di Fisiologia Veterinaria e Cattedra di Fisiologia Generale e Speciale Veterinaria II e Fisica Biologica, Università degli Studi di Bologna, Via Belmeloro, 8/2 Bologna and *Esercito Italiano, Centro Militare Allevamento e Rifornimento Quadrupedi, Grosseto, Italy*

Summary. Peripheral plasma levels of oestrone sulphate, androgens and LH were studied in two mating stallions. A peak of oestrone sulphate was observed 7.5–15 min after exposure to an oestrous female. No variations were recorded in LH concentrations. The concentrations of testosterone, dihydrotestosterone and androstenedione increased similarly after mating and on a day of sexual inactivity.

Introduction

The ability of the stallion testis to synthesize androgens, such as testosterone, dihydrotestosterone and androstenedione, and normally to produce very large quantities of oestrogens has been well established (Nyman, Geiger & Goldzieher, 1959; Savard & Goldzieher, 1960; Lindner, 1961; Velle, 1966; Ganjam & Kenney, 1975). Conjugated oestrogens such as oestradiol-17 β sulphate and oestrone sulphate have also been identified in stallion testicular tissue (Raeside, 1969).

The patterns and levels of plasma androgens and oestrogens have been investigated over 24-h periods in different seasons of the year and in artificial photoperiods. However, the results are widely contrasting, especially with regard to the plasma level changes of these hormones (Cox, Williams, Rowe & Smith, 1973; Cox & Williams, 1975; Kirkpatrick *et al.*, 1976; Sharma, 1976; Kirkpatrick, Wiesner, Kenney, Ganjam & Turner, 1977; Thompson, Pickett, Berndtson, Voss & Nett, 1977; Thompson, Pickett & Nett, 1978; Raeside, 1978/1979; Ganjam, 1979; Raeside, George, Roskopf & Bosu, 1980). Plasma LH concentration has been related to seasonal or photoperiodic changes as well as to sexual behaviour and seminal characteristics (Thompson *et al.*, 1977).

The variations in plasma LH and androgens in horses during sexual activity have been determined (Cox & Williams, 1975; Ganjam & Kenney, 1975).

The aim of the present study was to examine the variations in plasma concentration of LH, androgens and oestrone sulphate in stallions with respect to sexual stimulation.

Materials and Methods

Animals

Two stallions (A, Thoroughbred and D, Anglo-Arab), aged 7 and 9 years were used between February and May.

Blood samples were always taken between 08:00 and 14:30 h. Jugular vein blood was

collected by venepuncture from each stallion into heparinized tubes and was centrifuged immediately at 2300 *g* for 10 min at 4°C. Plasma was removed and stored at -20°C until assay.

Experimental design

Seven plasma samples from each stallion were taken on a day when there was not sexual activity (i.e. non-mating day). Subsequently, the animals were allowed to mount an oestrous mare (mating days), and on each of the 4 days a sample was taken just before exposure to the mare, at exposure (08:30 h) and then at 7½, 15 and 30 min and 1, 2, 4 and 6 h.

Hormone assays

Androgens. Radioactive tracers, about 2000 d.p.m. of testosterone, dihydrotestosterone and androstenedione, were added to 0.5 ml plasma, which was then extracted with 7 ml diethyl ether. The ether extract was dried and the residue was redissolved in 200 µl diethyl ether and placed onto a silica gel plate. Partition chromatography with a solvent system of chloroform:ethyl acetate (85:15 v/v) was performed as described by Paradisi, Lodi, Bolelli & Venturoli (1980). The fractions containing the three androgens were dried in a vacuum and the residue was redissolved in 500 µl 0.05 M-PBS-EDTA, pH 7.5, containing 0.1% BSA. Aliquots of 100 µl were taken in duplicate for RIA. A 200 µl sample was placed into a scintillation vial and counted. Recovery was $72.7 \pm 1.29\%$ for [1,2,6,7-³H]testosterone, $78.6 \pm 1.64\%$ for [1,2,3-³H]dihydrotestosterone and $75.6 \pm 1.26\%$ for [1,2,6,7-³H]androstenedione. The blank value obtained by extracting, under the same conditions, an equal amount of double-distilled water was 4.12 ± 0.45 pg testosterone/tube, 2.81 ± 0.43 pg dihydrotestosterone/tube and 3.48 ± 0.68 pg androstenedione/tube. The intra-assay and inter-assay precisions were expressed as the coefficient of variation and were respectively: for testosterone, 5.1 and 10.1%; for dihydrotestosterone, 7.8 and 5.6%; and for androstenedione, 6.7 and 11.0% (all values based on 10 assays).

LH. A heterologous radioimmunoassay using an anti-ovine LH serum in combination with labelled ovine LH was developed as described by Bono, Gaiani & Tamanini (1979). The antiserum was raised in a rabbit to NIH-LH-S20 and used at a final dilution of 1:40 000. Ovine LH (LER-1374A) was radioiodinated with ¹²⁵I to a specific activity of 50–100 µCi/µg. Highly purified equine LH (LER-958-1) was used as standard in the assay. The antiserum showed a 35.7% cross-reaction with equine FSH (LER-1686-2). The sensitivity of assay, defined as the mass of hormone required to suppress the binding of the labelled hormone to 90% of the binding achieved with no hormone added (B/Bo), was 266.9 ± 36.9 (s.e.m.) pg/tube. The intra- and inter-assay precisions for replicate determinations of a pool of plasma were always <10%.

Oestrone sulphate. The antiserum for the heterologous radioimmunoassay was raised in rabbits to oestrone-3-hemisuccinate-BSA and used at a working dilution of 1:20 000. Cross-reactions (%) were: oestrone, 100; oestrone sulphate, 20; oestrone glucuronide, 18; oestradiol-17α and oestradiol-17β, 0.4; oestriol, <0.01. The antigen was [2,4,6,7-³H]oestrone, used at 80 000 d.p.m./ml. The plasma sample was diluted 1:50 (v:v) with 0.05 M-PBS-EDTA and 1 ml aliquot was extracted with 5 ml diethyl ether by vortexing for 30 sec. The supernatant containing the unconjugated oestrone was discarded. Then 100 µl antigen and 100 µl antiserum were added to 100 µl diluted plasma and incubated overnight at 4°C. Separation of free from bound hormone was obtained by adding 1 ml 0.3% dextran-coated charcoal and by centrifugation at 3200 *g* for 15 min at 4°C. A standard curve was produced with known amounts of oestrone sulphate (15.6, 31.2, 62.5, 125, 250, 500, 1000 and 2000 pg). The recovery of oestrone sulphate was calculated by previously adding ~1500 d.p.m. [6,7-

^3H oestrone sulphate to the plasma. Accuracy and precision were determined by adding known amounts of oestrone sulphate to equine plasma pools. The reliability of the method was as follows (values are mean $\pm$ s.e. for the no. of samples in parentheses).

Accuracy and precision

Stallion plasma pool (ng/ml)	247.8 $\pm$ 10.2 (10)
Stallion plasma pool + 50 ng/ml	326.9 $\pm$ 3.5 (10)
Stallion plasma pool + 250 ng/ml	506.1 $\pm$ 17.2 (10)
Castrated male plasma pool (ng/ml)	0.23 $\pm$ 0.1 (10)
Castrated male plasma pool + 25 ng/ml	19.7 $\pm$ 1.4 (10)
Castrated male plasma pool + 250 ng/ml	231.6 $\pm$ 4.5 (10)
Inter-assay precision (%)	7.3 (10)
Intra-assay precision (%)	4.1 (10)
Recovery (%)	91.4 $\pm$ 0.6 (34)
Blank (pg/tube)	3.3 $\pm$ 2.3 (10)
Sensitivity (pg)	25.7 $\pm$ 3.1 (10)

The reliability of the method was further assessed as follows. The equine plasma was diluted 1 : 50 (v:v) with 0.9% (w/v) NaCl and a 1 ml aliquot was extracted with 5 ml diethyl ether. The supernatant was then discarded. To the residue 1 ml 0.2 M-acetate buffer, pH 4.2, containing 250 units sulphatase (Sigma, Type H-I) was added and the mixture incubated overnight at 37°C. The aqueous material was extracted with 10 ml diethyl ether and the extract evaporated. The dried residue was dissolved in 200 μ l benzene:methanol (85:15 v/v) and transferred onto a Sephadex LH-20 microcolumn (7.5 $\times$ 0.5 cm). Chromatography and radioimmunoassay were performed as described by Seren, Bono & Bolelli (1976). A standard curve was produced with 3.9, 7.8, 15.6, 31.2, 62.5, 125, 250 and 500 pg oestrone. To correct for the actual amounts of oestrone sulphate these values should be multiplied by 1.38. The values for oestrone sulphate in two equine plasma pools assayed by the two methods (without and with enzymic hydrolysis respectively) were 221.3 $\pm$ 6.5 (n = 6) and 225.2 $\pm$ 9.1 (n = 6) ng/ml for Pool A and 268.2 $\pm$ 8.8 (n = 6) and 284.8 $\pm$ 7.0 (n = 6) ng/ml for Pool B.

Patterns of mating behaviour

Some behavioural patterns in the two mating stallions were also investigated in relation to arousal (courtship, vocalizations, lipcurl and intromission latency) and consummation (intromission frequency and ejaculatory frequency).

Results

The concentration of testosterone in the plasma samples of the two stallions on the non-mating day ranged from 390 to 670 pg/ml in the morning and from 1300 to 1700 pg/ml in the early afternoon. A similar pattern for testosterone was observed on mating days, although on such days peak testosterone values occurred 5 times out of 8 at 12:30 h and were lower thereafter, although they were maintained at quite high levels. On the other 3 occasions, the testosterone concentration continued to rise over the entire experimental period. The highest levels of testosterone on the 4 mating days were 1261, 2134, 1403 and 1041 pg/ml in Stallion D and 1318, 987, 1759 and 3006 pg/ml in Stallion A.

Androstenedione and dihydrotestosterone followed the same pattern as testosterone, although at lower concentrations. The testosterone:androstenedione and testosterone:dihydrotestosterone ratios were 2:1 and 6:1, respectively. The correlation coefficients for the three androgens examined were 0.86 for testosterone and androstenedione, 0.8 for testosterone and dihydrotestosterone and 0.68 for androstenedione and dihydrotestosterone in Stallion A and 0.91, 0.81 and 0.71 in Stallion D.

Cyclic fluctuations in LH levels were found in each stallion on the non-mating day as well as on the days when they mounted. Only occasionally did an increase occur after exposure to the female. In Stallion D, the levels of LH were generally higher than in Stallion A, the highest and lowest LH levels being 38–145 and 13–59 ng/ml, respectively.

Oestrone sulphate levels (Text-fig. 1) on the non-mating day ranged from 156 to 263 ng/ml. Each animal exhibited a peak level of this steroid within $7\frac{1}{2}$ min after exposure to an oestrous female (15 min on only one occasion). The maximum value of oestrone sulphate was 505 ng/ml in Stallion D and 428 ng/ml in Stallion A. These levels had returned to basal values within 30 min after exposure.

Text-fig. 1. Plasma concentrations of oestrone sulphate in two stallions on (a) a day of sexual inactivity and (b, c, d, e) on 4 days when mating (arrow) was allowed. The time of exposure to the mare (08:30 h) was taken as time 0.

Moreover, the two stallions exhibited marked differences in sexual behaviour. In Stallion A but not in Stallion D arousal was always characterized by the typical signs, but no differences were observed between the stallions in intromission latency and intromission frequency. Ejaculation latency was longer in Stallion A (60–70 sec) than in Stallion D (20–30 sec).

Discussion

On the four mating days a gradual increase in peripheral plasma androgens, and especially in testosterone, was observed in both stallions 2–4 h after exposure to the oestrous female and mating, which took place at 08:30 h. Ganjam & Kenney (1975) reported increased androgen concentrations in stallions 5 and 15 min after female exposure, whereas Cox & Williams (1975) were unable to detect any changes in testosterone concentrations in response to sexual stimulation. In the present study, androgens also increased on the non-mating day between 12:30 and 14:30 h, i.e. 2–4 h later than the increase observed after mating. The changes in androgen concentrations observed here might well depend on the diurnal variations of these hormones, although Ganjam (1979) could not detect any diurnal variations of plasma androgens in the horse. Too few data are available to establish with sufficient reliability the extent to which the variability found in this study can be attributed to sexual excitement.

The concentration changes of oestrone sulphate were unusual. On the non-mating day the plasma concentrations in both stallions were extremely high, nearly 100-fold higher than those of androgens. This is in agreement with previous findings (Raeside, 1978/1979; Raeside *et al.*, 1980). However, exposure to an oestrous mare and mating produced a rise in oestrone sulphate levels with peak values 7.5–15 min after exposure. According to Raeside (1978/1979), oestrone sulphate could provide a sensitive index of testicular endocrine function in the stallion, because there is a close correlation between plasma levels of oestrone sulphate and oestrogens and the seasonal changes of sexual activity in the stallion (see also Thompson *et al.*, 1977). The biological significance of oestrone sulphate can only be conjectured at present. In this context, as oestrogens and oestrone sulphate, synergistically with testosterone, regulate accessory sex gland activity in castrated boars (Joshi & Raeside, 1973) and testicular production of oestrone sulphate in the stallion is even greater than in the boar, one can suppose that hormonal control of sex gland activity may include oestrogen of testicular origin in the stallion too. Oestrone sulphate may also be involved in maintenance of libido in the stallion, since oestradiol is able to restore libido in geldings (Thompson, Pickett, Squires & Nett, 1980). The role of oestrone sulphate in the stallion requires further study.

In our investigation, sexual stimulation had no significant effect on plasma LH levels. However Convey, Bretschneider, Hafs & Oxender (1971) and Smith, Mongkonpunya, Hafs, Convey & Oxender (1973) were unable to confirm the rise in LH levels after ejaculation in bulls that was reported by Katongole, Naftolin & Short (1971) and no increase in LH after mating has been observed in the rabbit (Hilliard, Pang, Penardi & Sawyer, 1975).

Finally, the differences in sexual behaviour reported in the two stallions did not seem to be reflected in differences in the magnitude of androgen and oestrone sulphate variations.

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