Testosterone in Normal, Cryptorchid and Castrated Male Horses

J. E. COX
J. H. WILLIAMS
Department of Veterinary Clinical Studies, University of Liverpool

P. H. ROWE
Unit of Reproductive Biology, University of Liverpool

J. A. SMITH
Department of Veterinary Surgery, University of Bristol

A RECURRING FEATURE of veterinary surgery is the horse which behaves like a stallion but has no visible or palpable testicles. Many such animals are cryptorchids, but a proportion—sometimes called "false rigs"—are found at surgery to have been already castrated.

We wondered if these false rigs have an extra testicular source of androgens or whether their behaviour patterns were acquired before castration and have persisted in the absence of androgens.

If "false rigs" do have an extra-testicular source of androgens, could this source be the spermatic cord and epididymis? There is a belief amongst horse owners (recorded by O'Connor, 1950) that horses "cut-proud", i.e. are castrated below the level of the epididymis leaving the latter in position, retain more of their virility and that occasionally such horses behave like cryptorchids retaining all their stallion propensities.

If, however, "false rigs" do not have an extra-testicular source of androgen or do not differ from animals known to be geldings in their concentration of circulating androgens, could we devise a simple test based on blood samples which would enable us to distinguish the castrated horse from one with testicular tissue?

If such a test could be established and the premises on which it is based were valid, it would obviate the need for surgical intervention in "false rigs".

Nieschlag and Ishmael (1970) recorded that castration of the human male reduced testosterone concentration by about one sixth and that whereas the intramuscular injection of 5000 i.u. of Human Chorionic Gonadotrophin (HCG) stimulated a three fold increase in testosterone concentration in normal men, such an effect was not noted in the castrate. Lindner (1961) observed that intravenous injection of Human Chorionic Gonadotrophin (HCG) into two stallions at 5 i.u./kg and 15 i.u./kg respectively produced a rise in testosterone concentration in the spermatic vein within 30 minutes. Katangole, Naftolin and Short (1971) measured testosterone concentration in one bull given 500 i.u. of HCG. The concentration of testosterone was lowest at the time of HCG injection and started to rise 30 minutes later to reach a plateau about two hours after injection.

The present study is the result of an investigation of testosterone production by the male horse in which we looked at the basal concentrations of testosterone in peripheral plasma and at the effect of a large dose (12000 i.u.) of HCG on plasma testosterone concentrations in normal, cryptorchid and castrated male horses.

MATERIALS AND METHODS

(a) Animals. The test animals used were a normal three year-old Welsh Mountain Pony stallion and four other mature stallions, 15 known geldings and a total of 29 horses referred as rigs.

Blood samples were taken by jugular venepuncture into heparinised tubes and the plasma which was obtained by centrifugation was stored at -20°C prior to analysis. Results obtained from serum are consistently higher than from plasma and reliance cannot always be placed on results from plasma samples kept above 0°C for long periods of time.

(b) Reagents and materials. Antiserum to a testosterone — 3-Keto- Carboxymethoxy-oxime — bovine serum albumin conjugate was prepared in the rabbit, treated with BSA and Rivanol as described by Exley, Johnson and Dean (1971), and diluted to 1 in 10,000 in 0.1 M phosphate buffer (pH 7.0) containing 0.9 per cent sodium chloride, 0.01 sodium merthiolate and 0.1 per cent gelatin (Tesco, U.K.). This diluent is henceforth referred to as PBSM—0.1 per cent gelatin. The diluted antibody was stored at 4°C.

Analar grade ethanol was double distilled over calcium hydride before use, and Analar grade diethyl ether was shaken with ferrous sulphate and then freshly redistilled before use.

Testosterone—1,2,6,7—3¹H (sp. act. = 100 Ci/m mole)* and non-radioactive steroids were diluted in ethanol and stored at 4°C. An aliquot of the tritiated testosterone

* Radiochemicals.
solution was evaporated to dryness under a stream of nitrogen and rediluted in PBSM-0.1 per cent gelatin to about 260,000 dpm/ml before use in the radio-immunoassay.

The antibody and tritiated testosterone concentrations were selected to give optimum conditions for a standard curve ranging from 0 to 200 pg of non-radioactive steroid.

0.25 per cent Norit A Charcoal† and 0.0025 per cent Dextran-T40‡ in PBSM-0.1 per cent gelatin provided the dextran-coated charcoal suspension for the separation of "bound" and "unbound" steroid. This suspension was freshly prepared and used at 4°C.

Extraction of steroids from plasma and the radio-immunoassay itself were performed in glass tubes which were carefully washed and dried between assays.

The liquid scintillation medium contained 0.3 per cent PPO (2,5-diphenyloxazole) and 0.03 per cent dimethyl POPOP (1,4-bis-(4-methyl-5-phenyloxazoly 1)-benzene) dissolved in toluene. Samples were thoroughly shaken and allowed to stabilize before counting.

(c) Extraction. Aliquots of plasma ranging from 20 μl to 2 ml were extracted with 2 x 2 ml diethyl ether. After centrifugation, the ether extracts were transferred to the assay tubes and evaporated to dryness. The dried extracts were used for assay without further purification and no correction was made for losses which occurred during extraction, recovery being of the order of 85 per cent. Each sample was analysed in duplicate.

(d) Standard curve. Aliquots of a standard solution of testosterone containing 0, 20, 50, 100 and 200 pg were added in triplicate to assay tubes from which 4 ml of ether had been previously evaporated, and dried under a stream of nitrogen.

(e) Radioimmunoassay. After the preparation of the standards and experimental samples, 100 μl of the antibody solution was added to each assay tube, mixed and allowed to stand at room temperature for 30 min. 100 μl of the tritiated testosterone solution was then added, the tubes shaken briefly and incubated for 16-20 hr. at 4°C.

After incubation the separation of the antibody-bound and free steroid was performed at 4°C by the addition of 1.0 ml of the dextran-coated charcoal suspension to each tube.

After mixing, the tubes were allowed to stand for 10 min. before centrifugation at 1100xg for 10 min. at 4°C. The supernatants containing the antibody-bound steroid were decanted into scintillation vials and counted in 10 ml of scintillation fluid at a tritium counting efficiency of 47 per cent.

The testosterone content of each experimental sample was determined from the standard curve and the data expressed as pg/ml of plasma. This assay system is similar to that described by Hotchkiss, Atkinson and Knobil (1971).

\[ \text{FIG. 1. Welsh Mountain pony stallion, Taffy: concentrations of testosterone in peripheral plasma during the day.} \]

(f) Specificity. The antibody was checked for cross-reaction with other steroids. Dihydrotestosterone (17β-hydroxy-5α-androstan-3-one) had a significant cross reaction at 28 per cent whilst all other steroids tested had cross reactions less than 0.5 per cent.

(g) Precision. The mean variation of the duplicate analyses of 220 plasma samples whose testosterone concentration varied from 10 pg/ml to 7000 pg/ml was 11.3 per cent. The range of each duplicate analysis is indicated in the text figures by a vertical bar.

RESULTS

(a) Plasma testosterone concentration in the intact stallion

In the normal three year-old Welsh Mountain Pony stallion, Taffy, concentrations fluctuated widely throughout the day (fig. 1). Basal concentrations in this stallion varied from 80 to 1600 pg/ml. Samples taken at random from four other mature stallions gave concentrations of 65, 200, 300 and 1150 pg/ml respectively.

(b) Effect on testosterone concentration of HCG injection

Fig. 2 shows that HCG injection on three separate occasions into the stallion Taffy provoked a rapid increase in testosterone concentration which was followed by a decrease. It can be seen that on all three occasions, the concentration in the 25-35 min. period after HCG injection was considerably higher than the concentration recorded immediately prior to HCG.

† Sigma & Co. Ltd.
‡ Pharmacia.
(c) **Plasma testosterone concentration in the gelding**

In 15 horses known to be geldings, testosterone concentration measured in single samples from each animal averaged 15.3 pg/ml (S.D. ± 4.9 pg) and in two of these the highest concentrations recorded in the 78 minutes following HCG injection were 49 pg and 22 pg respectively.

(d) **Plasma testosterone concentrations in rigs**

Fig. 3 shows the testosterone concentration in peripheral plasma following HCG administration recorded in four horses presented as rigs. All four proved upon later surgical investigation to have at least one testicle present and retained—Case No. 1531 had one right abdominal testis, No. 1541 a left scrotal and a right inguinal testicle, No. 1528 one right abdominal testicle and No. 1540 a left abdominal and a right scrotal testicle. With the exception of Case No. 1528, there was a marked rise in testosterone concentration within 25-35 minutes of HCG injection.

A six year-old pony which was presented as a rig but on surgical investigation proved to have been already castrated was also tested. A sample taken immediately before HCG injection had 18 pg/ml of testosterone.
and eight samples taken in the following two hours averaged 12.6 pg/ml of testosterone (S.D. ± 1.4 pg).

A further 24 horses presented as rigs were tested before surgical investigation by taking two blood samples for analysis, one immediately prior to HCG injection, the second 25-35 min. later. The results obtained from these 29 horses are summarised in the Table.

Statistical analysis showed that hemi-castrates could not be distinguished from animals with two testes, suggesting that in hemi-castrates the remaining testis undergoes compensatory hypersecretion. This is supported by histological studies by one of us (JAS) which showed that the volume of Leydig tissue in retained testes in hemi-castrates is not different from that of a normal scrotal testis, whereas in animals with two testes, the volume of Leydig tissue in the retained testis is about half that in the contralateral scrotal testis.

### TABLE

**TESTOSTERONE CONCENTRATIONS IN HORSES PRESENTED AS RIGS**

<table>
<thead>
<tr>
<th>Animals previously castrated—&quot;False Rigs&quot;</th>
<th>Animals with testis or testes present—&quot;Cryptorchids&quot;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. Basal Concentrations in pg/ml</strong></td>
<td></td>
</tr>
<tr>
<td>Number</td>
<td>9</td>
</tr>
<tr>
<td>Mean</td>
<td>17.7</td>
</tr>
<tr>
<td>S.D.</td>
<td>±11.0</td>
</tr>
<tr>
<td>Range</td>
<td>10–43</td>
</tr>
<tr>
<td><strong>B. Concentration 25—35 Min. after i/v injection of 12000 i.u. of HCG in pg/ml</strong></td>
<td></td>
</tr>
<tr>
<td>Number</td>
<td>5</td>
</tr>
<tr>
<td>Mean</td>
<td>30.0</td>
</tr>
<tr>
<td>S.D.</td>
<td>±14.5</td>
</tr>
<tr>
<td>Range</td>
<td>14–57</td>
</tr>
<tr>
<td><strong>C. Rise in Concentration in pg/ml (i.e. B—A calculated for each animal)</strong></td>
<td></td>
</tr>
<tr>
<td>Number</td>
<td>5</td>
</tr>
<tr>
<td>Mean</td>
<td>6.20</td>
</tr>
<tr>
<td>S.D.</td>
<td>±6.65</td>
</tr>
<tr>
<td>Range</td>
<td>4–14</td>
</tr>
</tbody>
</table>

Accordingly in the Table the results from hemicastrates and entires have been combined. From these results, it can be shown that "false rigs" have basal concentrations of testosterone which do not differ significantly (P > 0.40) from those of known geldings but which do differ significantly from those of cryptorchids (P < 0.001). Concentrations 25-35 min. post HCG are also significantly different in "false rigs" and cryptorchids (P < 0.01).

Moreover, the injection of HCG does not provoke a significant rise within 25-35 minutes in "false rigs" (P > 0.10) but does so in cryptorchid animals (P < 0.05), although when data are grouped in this way, differences between individual animals are masked.

When animals are considered individually, there is a marked tendency for those cryptorchids with low initial concentrations to respond more to HCG than those with higher initial concentrations.

**DISCUSSION**

The studies of normal horses reported here show that it is different in several respects from other domesticated animals. The smoothly fluctuating diurnal variation in testosterone concentration shown by the stallion is unlike the spikey pattern reported of the bull and ram.
Of 29 horses presented as rigs all those which were later found to have been already castrated had plasma testosterone concentrations indistinguishable from those found in geldings (nine animals) and failed to respond to HCG injection (five animals).

Evidence is presented that the epididymis and spermatic cord of the horse are not capable of producing significant quantities of testosterone.

The possibility is discussed of using paired blood samples, one taken prior to and the other taken after HCG injection, as a test of whether or not a horse has been castrated.

SUMMARY

Concentrations of testosterone in the peripheral plasma of normal, cryptorchid and castrated male horses have been measured by a rapid radioimmuno-assay. Basal concentrations in horses with one or two testicles range from 65 pg to 1600 pg/ml. Intravenous injection of 12000 i.u. of Human Chorionic Gonadotrophin (HCG) into horses possessing testicular tissue stimulated a rise in testosterone concentration which could be detected within 25-35 minutes of the injection.

Geldings consistently showed low testosterone concentrations in the plasma (15.3±4.9 pg/ml; 15 animals), and injection of HCG did not stimulate a significant rise in testosterone concentration.

EQUINE VETERINARY JOURNAL

(Katangole et al., 1971) and observed in the rabbit and goat (Rowe, unpublished observations). This smooth fluctuation is more like the pattern seen in man (Lincoln, 1971).

It is interesting to speculate as to whether this difference is due to horse and man living in aural and visual contact with females of their own species rather than in artificial isolation like the bull, ram, rabbit and goat.

The concentrations recorded in the stallions are much lower than those recorded in the other species mentioned but it must be remembered that only free steroids were measured in our assay. Raeside (1969) has presented evidence that the stallion has primarily conjugated oestrogens in the testis and it may be that other steroids are present primarily as conjugates.

From the observations made in rigs it is apparent that the behaviour (such as attempting to serve mares and, in some cases, erection and intromission) which is reported of "false rigs" is not associated with higher concentrations of circulating testosterone than occur in geldings. This supports the contention that "false rigs" do not have a major extra-testicular source of androgens and suggests that their masculine behaviour might have been acquired before castration and have persisted in the absence of androgenic stimulation.

The possibility of using a simple blood test to determine whether or not a horse has been castrated is suggested by the results given in the Table. The test would require paired blood samples to be submitted for analysis for testosterone, one sample immediately prior to the intravenous injection of HCG and the other some time afterwards. Paired samples are required to allow a clear distinction to be made between geldings with relatively high concentrations and cryptorchids with relatively low basal concentrations, especially if samples have to be transmitted to the laboratory by post.

The optimum dose of HCG has yet to be determined since 12000 i.u. was chosen somewhat arbitrarily. The optimum time after HCG for the second sample to be taken is also open to discussion. In theory 60 minutes may be better than 30 minutes but the latter is likely to be more convenient.

The experiments described under "Effects of 'Cutting-Proud'" cast doubt upon the beliefs that this technique is a means of retaining androgen-producing tissue and that removal of a length of spermatic cord cures "false rigs" by removing a piece of androgen-producing tissue. Indeed, six of the "false rigs" which could be traced, were at least as difficult to handle after they had had their spermatic cords removed as they were before the operation, and one of them was still able to mount and to serve a mare.

RESUME

Les concentrations de testosterone dans le plasma paralitrique des chevaux males normaux, cryptorchides et castr6s ont et6 mesur6s par des tests radio actifs.

La concentration de base chez les chevaux avec un ou deux testicules varie de 65 pg a 1600 pg/ml.

L'injection intraveineuse de 12000 U.I. de gonadotrophine chorionique humaine chez des chevaux poss6dant un tissu testiculaire entrainait.

L'augmentation du taux de testost6r6ne detectable entre 25 et 35 minutes apr6s l'injection.

Les chevaux hongres montr6rent de basses concentr6s plasmatiques de testost6r6ne (15.3±4.9 pg—
15 sujets) et l'administration de gonadotrophine ne provoqua aucune 6levation significative du taux de testost6r6ne chez eux.

Des 29 chevaux reput6s cryptorchides tous ceux qui furent identifi6s plus tard comme ayant 6t6 castr6s montr6rent des concentrations plasmatiques de testost6r6ne de m6me ordre que les hongres, et ne present-6rent aucune r6ponse 6 l'injection de gonadotrophine.

Il semble que ni le lepididyme ni le cordon spermatique du cheval ne soient capables de secr6ter des quantit6s significatives de testost6r6ne.

On envisage la possibilit6, en comparant deux echantillons de sang, l'un preleve avant, l'autre preleve apr6s l'injection de gonadotrophine chorionique, d'6tablir si un cheval a 6t6 ou n'a pas 6t6 castr6.

ZUSAMMENFASSUNG


Wallachen zeigten immer tiefe Testosteronkonzentra-

tion im Plasma (15.3±4.9 pg/ml, N=15) und die Injektion von Choriongonadotropin führte nicht zu einem signifikanten Anstieg. Von 29 Pferden, die als Spitzhengste vorgestellt wurden, hatten alle diejenigen (9) tiefe Plasma-Testosteronkonzentration, die sich als schon kastriert herausstellten und sie sprachen auch nicht auf Choriongonadotropin an (5). Es wird gezeigt, dass Epididymis und Samenstrang beim Pferd keine signifikanten Testosteronmengen produzieren. Die Möglichkeit wird diskutiert, mit gepaarten Blutproben (vor und nach Choriongonadotropin-Injektion) abzuklären, ob ein Pferd kastriert worden ist oder nicht.
ACKNOWLEDGEMENTS

We wish to thank the veterinary surgeons who referred the rigs to our schools, Professor Messervey for operating on the horses at Bristol and Professor Fitzpatrick for the provision of facilities at Liverpool. We also gratefully acknowledge the generous financial support of the Horserace Betting Levy Board, Beecham's Research Laboratories and The Wellcome Trust.

REFERENCES


