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Concentrations of plasma total and unbound progesterone and testosterone during pregnancy in Cape porcupines, *Hystrix africaeaustralis*

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Total plasma and unbound progesterone and testosterone concentrations in pregnant Cape porcupines were determined by radioimmunoassay and centrifugal ultrafiltration dialysis of plasma collected at weekly intervals throughout gestation. During pregnancy, total plasma progesterone and testosterone concentrations attained values of up to ~200 ng/ml and ~2000 pg/ml, respectively. However, unbound progesterone and testosterone remained relatively constant at 2% and 13% of total values, respectively. The low concentrations of unbound steroids result from high concentrations of plasma binding proteins which probably protect progesterone and testosterone from metabolism during pregnancy. High testosterone concentrations may induce the production of plasma progesterone binding proteins during pregnancy to maintain high progesterone concentrations.

Key words: Progesterone; Testosterone; Pregnancy; *Hystrix africaeaustralis*.

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Introduction

Pregnancy in hystricomorph rodents is characterised by an increase in the plasma concentrations of progesterone-binding globulin (PBG) associated with the formation of a definite placenta (Heap *et al.*, 1981). The high affinity of PBG for progestogens and androgens (Diamond *et al.*, 1969; Milgrom *et al.*, 1970; Stroupe and Westphal, 1975; Louw *et al.*, 1992) apparently retards their metabolism, thereby increasing plasma concentrations (Westphal *et al.*, 1977; Heap *et al.*, 1981; van Aarde and Potgieter, 1986). Plasma progesterone concentrations in the Cape porcupine, *Hystrix africaeaustralis*, are relatively low (~20 ng/ml) during the initial stages of the 94 day gestation period, increasing rapidly thereafter to reach maximum values (102–180 ng/ml), 50–60 days after mating (van Aarde and Potgieter, 1986). The trend and concentrations are similar to those reported for other hystricomorph rodents (see Heap *et al.*, 1981).

The generally accepted model of steroid hormone action proposes that only the steroid in circulation which is not bound to serum proteins can diffuse into target cells and bind to intracellular receptors (Siiteri *et al.*, 1982; Rosner, 1990). Thus, steroid–protein complexes in the blood are assumed to be inert (Klosterman *et al.*, 1986). However, it has been speculated that both unbound and bound steroids are biologically active (Pardridge, 1988).

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To the best of our knowledge no distinction has as yet been made between the unbound and bound fractions of progesterone in hystricomorph rodents. However, in this regard some information is available for testosterone in guinea-pigs (Rigaudiere *et al.*, 1980). The present study quantifies changes in concentrations of plasma and unbound progesterone and testosterone throughout pregnancy in the Cape porcupine, an Old World hystricomorph rodent with reproductive characteristics similar to that of the New World hystricomorph rodents (see van Aarde, 1985a).

Materials and Methods

Statistics

Values are expressed as means ($\bar{x}$) followed by one standard deviation ($\pm$SD) of the mean. Student's t-test values are used to determine statistical significance of differences between means at the 5% level. Student's t-test on percentage values are based on the arc-sine transformation of the relevant data. In the analyses of variance, Tukey multiple range tests were used to determine the variables that differed significantly.

Animals and blood sampling

Porcupines were housed in semi-outdoor concrete enclosures at the University of Pretoria's Experimental Farm (25°45′S, 20°12′E) as described by van Aarde (1985a). Enclosures were inspected daily for the presence of new-born animals and the date of conception was calculated as the date of birth minus the mean gestation length (94 days; van Aarde, 1985b).

Pregnant females were immobilized at weekly intervals throughout pregnancy by an intramuscular injection of a mixture of xylazine hydrochloride ("Rompun", Bayer, Johannesburg, South Africa: 0.38 mg/kg) and ketamine hydrochloride ("Ketalar", Parke-Davis Laboratories (Pty) Ltd., Isando, South Africa: 0.75 mg/kg). Blood ($\sim$5.0 ml) was collected from the femoral vein into heparinised tubes and was centrifuged for 10 min to remove erythrocytes. Plasma was stored at -20°C until assayed and aliquots used for dialysis were centrifuged (1500 ***g*** for 5 min) after thawing to remove insolubles before assaying.

Determination of steroid concentrations

Progesterone. Plasma progesterone concentrations were determined by radioimmunoassay (RIA) as described by van Aarde and Potgieter (1986) and cross-reactivity of the antiserum is provided in van Aarde and van Wyk (1991). Recovery of [1,2,6,7-^{3}H] progesterone in 0.1 ml plasma pools averaged 81.7% ($N = 6$). Buffer blanks gave values less than the assay sensitivity (0.1 ± 0.03 ng/ml; $N = 6$). The inter-assay coefficient of variation was 2.96% ($N = 6$) with an intra-assay coefficient of variation 1.4%.

Testosterone. Total plasma testosterone levels were determined by RIA as described by van Aarde and Skinner (1986) after denaturation of binding proteins by the addition of a 0.1 ml sodium hydroxide solution (0.6 M) to 0.1 ml plasma aliquots. Testosterone antibody was donated by R. P. Millar (Department of Chemical Pathology, University of Cape Town, Cape Town, South Africa) and cross-reactions with naturally occurring steroids were less than 0.1%, except for dehydrotestosterone for which it was 5.1%. The sensitivity of the assays was 9.9 ± 4.6 pg/ml ($N = 4$). The inter-assay coefficient of variation was 4.2% ($N = 4$) and intra-assay coefficient of variation was 1.5%. Recovery of [1,2,6,7-^{3}H] testosterone in 0.1 ml plasma pools averaged 93.9% ($N = 4$). Serially diluted plasma samples pooled from pregnant females yielded displacement curves parallel to the standard curve.

Determination of concentrations of unbound fractions of plasma progesterone and testosterone

Centrifugal ultrafiltration dialysis. Preparation of ultrafiltration units: ultrafiltration units were prepared as described by Hammond *et al.* (1980). Each unit comprised a scintillation vial [diameter 13 mm; Mini Poly-Q vials; Beckman Instruments (Pty) Ltd., Johannesburg, South Africa], and a glass cylinder (internal diameter, 10 mm). One end of the glass cylinder was covered with a wet dialysis membrane (M_r cutoff $\leqslant$10 000; Labretoria, Pretoria, South Africa) which was spread evenly and held in position by two elastic bands. Three filter paper discs (diameter 13 mm;

Whatman No. 1; Saarchem, Krugersdorp, South Africa) were placed at the bottom of the scintillation vial. The prepared glass cylinder was blotted dry before placing it into the scintillation vial.

Experimental procedure. The method used was, with minor modifications, similar to that described by Hammond *et al.* (1980). [1,2,6,7-^{3}H] Progesterone (0.93 nM; Code TRK 413, spec. act. 96 Ci/mmol; Radiochemical Centre, Amersham, U.K.) or [1,2,6,7-^{3}H] testosterone (1.13 nM; Code TRK 402, spec. act. 80 Ci/mmol: Radiochemical Centre, Amersham, U.K.) in 0.5 ml ethanol were dried at 37°C in glass extraction tubes under a stream of nitrogen. This was followed by the addition of 0.005 ml (16.5 pM) D-[U-^{14}C] glucose (Code CFB 96, spec. act. 270 mCi/mnol; Radiochemical Centre, Amersham, U.K.) in distilled water and 0.45 ml plasma to each tube. These tubes were vortexed for 5 sec, and incubated under an atmosphere of 95% O_2:5% CO_2 in a waterbath at 37°C for 30 min and thereafter for 30 min at room temperature (~24°C) and environmental atmospheric conditions. An aliquot (0.2 ml) of this mixture was pipetted onto the dialysis membrane of two ultrafiltration units. The glass cylinders were then placed in the scintillation vials prepared as described above, capped and centrifuged (1500 ***g***) for 1 hr at 25°C. During centrifugation, an ultrafiltrate of 0.051 ± 0.005 ml ($N = 6$) accumulated on the filter paper discs in the scintillation vials. After centrifugation, the glass cylinders were carefully removed from the scintillation vials and 0.05 ml of the contents remaining inside the glass cylinder were pipetted onto filter paper discs at the bottom of another scintillation vial. Distilled water (0.35 ml) was added to all the vials and the contents vortexed. After the addition of scintillation fluid (5 ml) to the vials, they were capped and left for 12 hr before counting (to allow the radioactivity to disperse from the filter paper into the scintillation fluid) in a Packard 1500 Tri-carb liquid scintillation counter set for dual counting.

Calculation of the percentage unbound steroid. Differences in the ratios of radioactivity in the aliquots collected from the glass cylinders after centrifugation and that in ultrafiltrates which accumulated on the filter paper discs during centrifugation, were used to determine the percentage unbound steroid as follows:

$$\% \text{ Unbound steroid} = \left(\frac{[^3\text{H}] \text{ ligand (dpm)}}{[^{14}\text{C}] \text{ glucose (dpm)}} \right)_{\text{(plasma)}} - \left(\frac{[^3\text{H}] \text{ ligand (dpm)}}{[^{14}\text{C}] \text{ glucose (dpm)}} \right)_{\text{(ultrafiltrate)}} \times 100$$

(from Hammond *et al.*, 1980).

The results are only valid provided the described experimental procedures will allow equilibration to be attained.

Validation of centrifugal ultrafiltration dialysis. The validity of using D-[U-^{14}C] glucose to monitor the movement of unbound steroids in porcupine plasma was assessed by adding 0.05 ml D-[U-^{14}C] glucose (0.165 nM) to 6 ml plasma and dialysing it overnight at 4°C against 20 ml phosphate-buffered saline (PBS; pH 7.0). Since concentrations of D-[U-^{14}C] glucose in the plasma and dialysis buffer were equal it could be concluded that D-[U-^{14}C] glucose was evenly distributed on both sides of the dialysis membrane and that equilibrium has been reached. It also illustrated that D-[U-^{14}C] glucose did not bind to plasma components and reflects the movement of unbound components in plasma.

To demonstrate that the ratio of unbound [^{3}H] steroid:[^{14}C] glucose in the ultrafiltrate was not altered during experimental procedures, 6 ml plasma was dialysed overnight against 20 ml PBS at 4°C. Treatment of the PBS dialysate as described under 'Experimental procedure' showed that the ratios of steroids remained nearly identical on both sides of the dialysis membrane in the ultrafiltration unit (ratio = 0.99 ± 0.17 ($N = 6$) and 0.90 ± 0.08 ($N = 6$) for progesterone and testosterone, respectively). In the absence of binding proteins, the unbound fraction thus moved freely through the membrane during centrifugation.

The recoveries of [1,2,6,7-^{3}H] progesterone, [1,2,6,7-^{3}H] testosterone and D-[U-^{14}C] glucose from distilled water under experimental conditions were 103.6 ± 5.51% ($N = 6$), 101.3 ± 2.15% ($N = 6$) and 85.2 ± 15.25% ($N = 6$), respectively, and from plasma 100.8 ± 3.93% ($N = 6$),

100.2 ± 5.1% ($N = 6$) and 94.0 ± 4.98% ($N = 6$), respectively. The concentrations of D-[U-^{14}C] glucose (165 pM) in plasma samples before and after centrifugal ultrafiltration were identical, indicating that no loss occurred due to adsorption to surfaces within the ultrafiltration unit during centrifugation.

Temperature during centrifugation did affect estimates of the percentage unbound steroid in samples. The percentage unbound progesterone increased significantly ($F_{1,11} = 7.72$; $P < 0.001$; Tukey multiple range test; $P < 0.01$) from 15°C (4.7 ± 0.31) to 25°C (6.5 ± 1.07) but the difference in values at 25°C and 37°C (6.7 ± 0.84) was not significant. The percentage unbound testosterone at 15°C (12.38 ± 2.04) and 25°C (13.74 ± 1.22) was similar, but significantly higher at 37°C (19.74 ± 3.31) than at 25°C ($F_{1,15} = 15.97$; $P < 0.001$; Tukey multiple range test; $P < 0.01$).

The estimated percentage unbound progesterone in plasma was not affected by the volume of the ultrafiltrate that formed after centrifugation for different periods at 25°C or the initial volume of the sample within the glass cylinder. Furthermore, after centrifugation at 1500 *g* for 1 hr at 25°C, unbound progesterone represented 5.2 ± 0.62% of the total progesterone compared to a value of 5.7 ± 2.08% when the ultrafiltration unit was left to stand for 16 hr at room temperature (24°C). Centrifugation thus apparently reduced variability but did not affect absolute values.

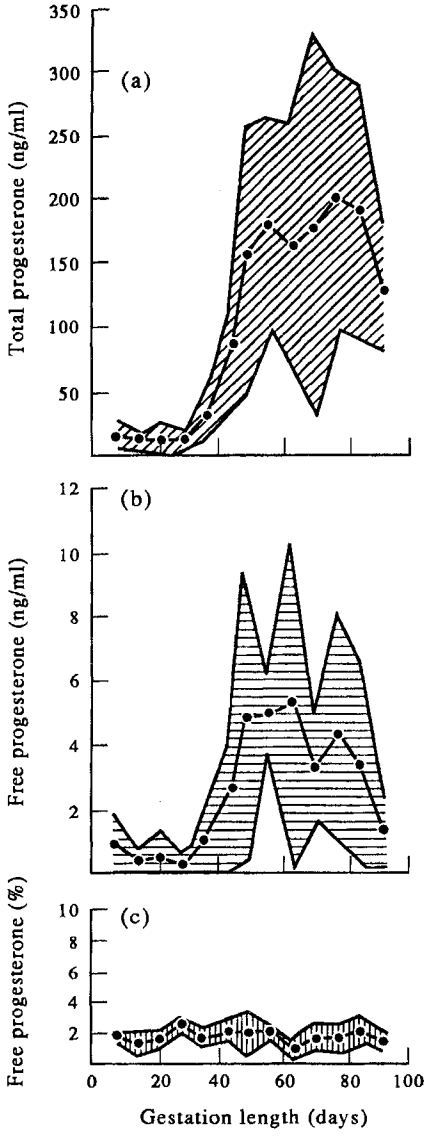

Fig. 1. Temporal changes in progesterone concentrations. Plasma progesterone levels (mean ± SD: shaded area) for 11 Cape porcupine females: (a) total progesterone concentration (ng/ml), (b) free progesterone concentration (ng/ml; calculated from the percentage free progesterone and total progesterone concentrations), and (c) free progesterone (%).

Results

Plasma hormone concentrations

Despite individual variability, plasma progesterone concentrations remained relatively low for the first 30 days of pregnancy, increasing rapidly thereafter to reach maximum concentrations between days 60 and 80 of pregnancy (Fig. 1a). Progesterone concentrations decreased rapidly during the last 15 to 30 days of pregnancy and returned to preconception levels (<7 ng/ml) within 1 week after parturition. Four of the 11 pregnancies for which complete profiles were obtained, were characterised by double peaks (days 40–50 and 60–80 of pregnancy) in plasma progesterone concentrations. Concentrations during the first 30 days of pregnancy ranged from 2.2 to 97.4 ng/ml ($\bar{x} = 15.9 \pm 21.34$; $N = 25$) while peak concentrations for these 11 pregnancies ranged from 102 to 451 ng/ml ($\bar{x} = 202 \pm 98$).

During pregnancy plasma testosterone concentrations followed a trend similar to that of progesterone concentrations. Peak values ranged from 0.64 to 7.99 ng/ml ($\bar{x} = 2.27 \pm 1.798$ ng/ml; $N = 11$) and

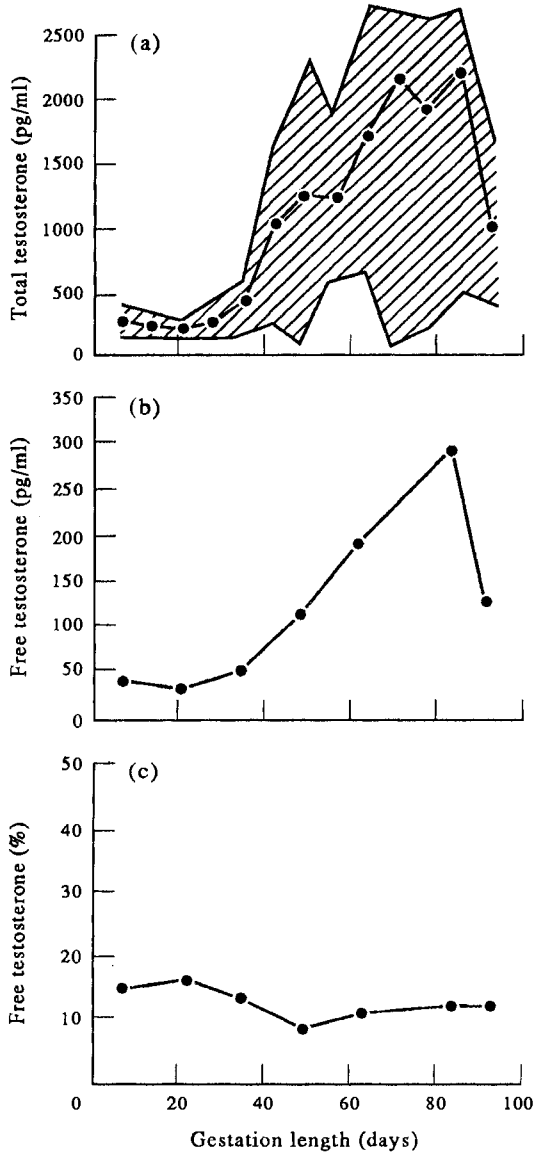

Fig. 2. Temporal changes in testosterone concentrations. Plasma testosterone levels (mean ± SD: shaded area where applicable) for pooled plasma of 11 Cape porcupine females: (a) total concentration (pg/ml) for individual animals, (b) free testosterone concentration (pg/ml in pooled plasma; calculated from the percentage free testosterone and total testosterone in pooled plasma), and (c) free testosterone (%).

concentrations during the first 30 days of pregnancy varied from 200 to 400 pg/ml ($\bar{x} = 269 \pm 122$ pg/ml; $N = 55$) (Fig. 2a).

Unbound hormone concentrations

Concentrations of unbound progesterone in plasma increased during pregnancy from <2 to 5.4 ± 5.38 ng/ml ($N = 11$) but represented only ~2% of total plasma progesterone concentration (Fig. 1c). The unbound concentrations increased with an increase in concentrations of total plasma progesterone (Fig. 1b).

Based on the analysis of pooled samples for different stages of pregnancy, percentage unbound testosterone remained relatively constant at 13.7 ± 1.22% at 25°C and 19.7 ± 3.31% at 37°C throughout pregnancy (see Fig. 2a–c). Concentrations of unbound testosterone remained low (<40 pg/ml) for the first 35 days of pregnancy, increasing rapidly thereafter to a peak value (297 pg/ml) at week 12 of pregnancy (Fig. 2c).

A highly significant correlation existed between concentrations of unbound and total plasma progesterone ($r = 0.92$; $P < 0.001$; $N = 13$), unbound and total concentrations of plasma testosterone ($r = 0.98$; $P < 0.001$; $N = 7$) and between plasma progesterone and plasma testosterone concentrations ($r = 0.93$; $P < 0.001$; $N = 13$) (Fig. 3).

Discussion

The concentrations of plasma progesterone recorded during pregnancy in the current study are similar to those of van Aarde and Potgieter (1986). High progesterone concentrations in hystricomorph rodents apparently result from the presence of PBG (Heap and Illingworth, 1974) and the formation of accessory corpora lutea (Tam, 1974) which is one of the outstanding features of the porcupine ovary (see van Aarde and Skinner, 1986). Considering that either the unbound or bound fraction of a steroid may enter target cells (Pardridge, 1988), measurements of total steroids alone may not provide an adequate evaluation of the biological action of a hormone.

The reasons for the high plasma concentrations of progesterone and testosterone in hystricomorph rodents still need to be established, but could be due to a decreased rate in metabolism (Heap and Illingworth, 1974) or the increased secretion of both, or to a combination thereof (Illingworth *et al.*, 1970). The high affinities of plasma progesterone binding proteins and high capacities of albumin and other plasma proteins for progesterone may give rise

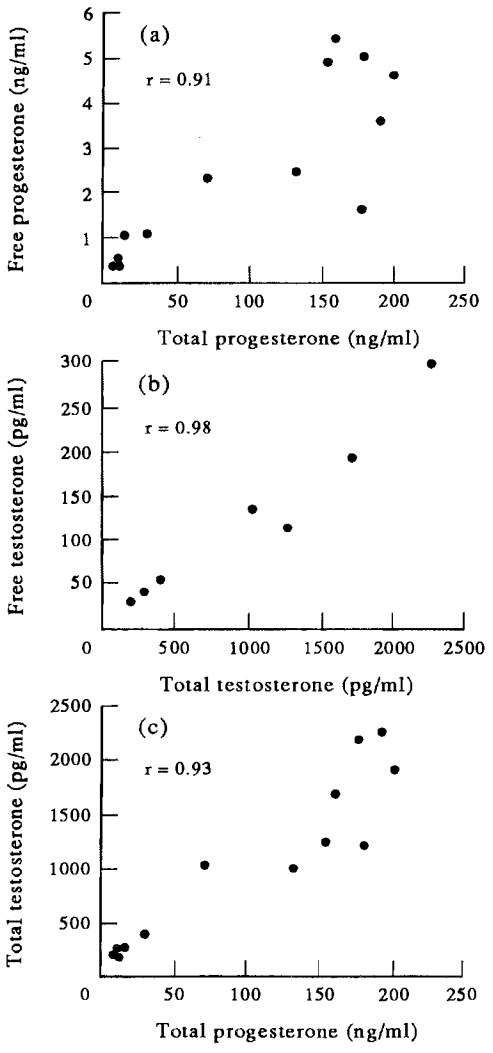

Fig. 3. The relationships between: (a) free and total concentrations of progesterone, (b) free and total concentrations of testosterone and (c) total progesterone and testosterone concentrations throughout pregnancy for undiluted pooled plasma of Cape porcupines. r = correlation coefficients.

to the low levels of unbound plasma progesterone in porcupines (see Louw *et al.*, 1992).

Plasma concentrations of testosterone in pregnant porcupines followed a trend similar to that of plasma progesterone, suggesting that there may be an interaction between these two steroids in their influence on gestation, the onset of parturition or maternal responsiveness. In porcupines, plasma concentrations of oestradiol-17β also follow a trend similar to that of progesterone (van Aarde and Skinner, 1986), which implies that some interaction may exist between the secretion or production of testosterone and oestradiol-17β. Gibori and Keyes (1978) showed that testosterone in pregnant rats is essential to maintain a high progesterone concentration and in guinea-pigs, plasma testosterone and dehydrotestosterone concentrations also increased significantly during pregnancy (Rigaudiere *et al.*, 1980).

In guinea-pigs, testosterone followed a trend similar to that recorded by us in the Cape porcupine, though absolute concentrations in our study are higher (Fig. 2) than those recorded for guinea-pigs. In pregnant guinea-pigs, testosterone apparently plays an important role in the metabolism of dehydrotestosterone and androstenedione (Despres and Rigaudiere, 1983). No information is as yet available on this relationship in porcupines.

Progesterone-binding proteins may be important to protect the guinea-pig mother from high unbound androgen concentrations during pregnancy (Diamond *et al.*, 1969). Testosterone injected from Day 18 *post coitum* stimulated progesterone-binding of plasma in pregnant guinea-pigs (22–26 days *post coitum*) and may thus be of significance in the production of progesterone binding proteins during pregnancy (Diamond *et al.*, 1969; Heap and Illingworth, 1974). However, it has also been postulated that other steroid-binding factors in conjunction with high progesterone concentrations may play a role in protecting the adult female from excess androgenic concentrations (Diamond *et al.*, 1969).

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