Use of a GnRH Agonist and hCG to Obtain an Index of Testosterone Secretory Capacity in the Koala (*Phascolarctos cinereus*)

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ABSTRACT: Testosterone secretion in mammals typically occurs in random pulses such that a single blood sample provides limited information on reproductive endocrine status. However, it has been shown in several species that an index of the prevailing testosterone biosynthetic capacity of the testes can be obtained by measuring the increase in circulating testosterone after injection of a GnRH agonist or human chorionic gonadotrophin (hCG). Hence, the aims of the present study were to examine fluctuations in testosterone secretion in the koala (n = 6) over a 24-hour period and then characterise testosterone secretion after injection of the GnRH agonist buserelin (4 μg) or hCG (1000 IU). The latter was used to establish an index of the prevailing testosterone biosynthetic capacity of the koala testis. Individual koalas showed major changes in blood testosterone concentrations over 24 hours, but there was no apparent diurnal pattern of testosterone secretion ($P > .05$). Injection of buserelin and hCG resulted in an increase ($P < .05$) in blood testosterone concentration. After injection of exogenous hormone, near maximal concentrations of testosterone occurred at around 60 minutes. There was a tendency for plasma testosterone to decline after 90 minutes with buserelin, but concentrations remained close to the upper limit for 240 minutes with hCG. There were strong positive correlations between the average testosterone concentration over 24 hours and the maximum observed testosterone concentration after stimulation with GnRH and hCG (GnRH, $r = .772$; $P = .07$ and hCG, $r = 1.0$; $P < .01$). The findings in the present study confirmed that individual male koalas can show large fluctuations in blood testosterone concentrations over time and that a GnRH agonist and hCG can be used in the koala to obtain an index of the prevailing steroidogenic capacity of the testes.

Key words: Marsupial, hormone stimulation test.

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Testosterone secretion in males typically occurs as random pulses, and a single measurement of blood testosterone is not a reliable indicator of reproductive endocrine status (Cleva et al, 1994; Stocco, 1999). To address the pulsatile nature of testosterone secretion, exogenous hormones such as GnRH, GnRH agonists, luteinizing hormone (LH), and human chorionic gonadotropin (hCG) have been used to induce an acute increase in testosterone secretion (Wildt, 1996). In several species, including men, maximal testosterone concentrations after the injection of a trophic hormone are reflective of testosterone secretion in the period before hormone injection (Bradley and Stoddart, 1997; Parlevleit et al, 2001; Kauschansky et al, 2002). The

testosterone response to exogenous trophic hormone stimulation, therefore, provides a diagnostic index of the prevailing steroidogenic capacity of the testes (Wildt, 1996). The availability of a practical testosterone stimulation test for male koalas would facilitate reproductive endocrine studies in this unique species.

McFarlane (1990) previously reported that treatment with 2 μg of natural sequence GnRH did not induce an increase in testosterone secretion in koalas. It is possible that the dose of GnRH used in the latter study was below the threshold required to induce LH and testosterone responses. This may have been related, in part, to rapid clearance of natural sequence GnRH from circulation. In the present study, a GnRH agonist with a longer half-life in circulation than natural sequence GnRH and higher affinity for the GnRH receptor was used to ascertain whether a testosterone response could be elicited with exogenous hormone in male koalas. Given that the testosterone response to GnRH relies on LH release from the pituitary, direct stimulation of the testes with hCG was also evaluated as a potential testosterone stimulation test in koalas.

The specific aims of the present study were to examine fluctuations in endogenous testosterone secretion in the koala (n = 6) over a 24-hour period and then to characterise testosterone secretion after injection of the GnRH agonist buserelin or hCG, in order to establish a reliable index of the prevailing testosterone bio-synthetic capacity of the koala testis.

Materials and Methods

Animals

Six sexually mature (>4 years old) koalas (K1–K6) were used in all experiments. The live weight of the animals was 6.5 ± 1.0 kg, and all animals remained clinically healthy throughout the experimental period. Three of the koalas (K1–K3) were wild sourced and temporarily located at the Queensland Parks and Wildlife Service Moggill Koala Hospital (MKH). The other 3 (K4–K6) were permanently housed at the University of Queensland Koala Study Program (KSP). The study was approved by the University of Queensland Animal Ethics Committee (SAS/386/04) and was conducted over a 3-week period during early spring 2004.

Anesthesia and Venipuncture Procedure

Venipuncture was conducted on anaesthetised koalas using a combination of chemical and gaseous agents (Blanshard, 1994; McGowan et al, 1994). Briefly, anesthesia was initially induced for the first blood sample by intramuscular injection of 20 μg of Zoletil 100 (tiletamine/zolazepam; Virbac, Peakhurst, Australia; Animal Health Australia, Canberra, Australia); subsequent blood samples for each experiment where recovered by induction with 2%–4% gaseous isoflurane (Attane; Pharmtech, Hornsby, Australia; Animal Health Australia) at a flow rate of 1.5 L of oxygen/minute.

Blood was collected from the cephalic vein using a 25 gauge winged infusion set (Miniset; Baxter Healthcare, Toongabbie, Australia) and 3-mL syringe (Terumo; Terumo Corporation, Binan, Philippines). Approximately 1 mL of blood was recovered at each venipuncture and dispensed into a 1.3-mL lithium-heparin micro tube (Sarstedt, Nümbrecht, Germany). Heparinised samples were maintained at room temperature for 5 minutes and then stored at 4°C until the end of sampling when they were centrifuged. To facilitate venipuncture, the forearm was shaved and a tourniquet applied at the elbow during sampling. Blood was collected from alternate arms and a cold pack was applied to the venipuncture site after each collection to minimise the risk of hematoma. Heparinized blood samples were centrifuged at 3000 × g for 10 minutes and plasma samples frozen at −20°C.

Experiment 1: Changes in Testosterone Secretion Over 24 Hours

In Experiment 1, each koala (K1–K6, n = 6) was bled at 0800, 1200, 1600, 2000, 0000, 0400, and again at 0800 hours. The objective in this experiment was to obtain a measure of the variability of the prevailing testosterone secretory status of individual koalas before undertaking stimulation tests with a GnRH agonist and hCG.

Experiment 2: GnRH Agonist Stimulation Test

The GnRH agonist test was conducted over 2 days, in which the KSP koalas (n = 3) were assessed on the first day and MKH koalas on the second day (n = 3). After the initial blood sample (T0) each koala received a 4-μg injection IM of the GnRH agonist buserelin (Receptal; Intervet, Bendigo, Australia). Other blood samples were taken at 30, 60, 90, 120, 180, and 240 minutes after injection of GnRH.

Experiment 3: hCG Stimulation Test

Experiment 3 was the same design as Experiment 2 except that 1000 IU human chorionic gonadotrophin (Chorulon; Intervet) were injected IM. MKH koalas (n = 3) were bled on the first day and the KSP koalas (n = 3) on the second.

Hormone Analysis

Plasma samples were assayed for testosterone concentration using SpectriaTestosterone RIA kits (Orion Diagnostica, Espoo, Finland). The assay was validated for koala plasma by demonstrating parallelism between dilutions of pooled plasma and the standard curve (F6, 28 = 1.87, P = .121). The intraassay and interassay coefficients of variation were 5.3% (n = 20; mean = 4.3 ng/mL) and 12.0% (n = 10; mean = 6.0 ng/mL), respectively. A recovery of 97.5% was obtained when testosterone was added to a plasma sample before assay. The detection limit of the assay was 0.07 ng/mL. All samples were assayed in duplicate. The testosterone antibody cross reacted with testosterone 100%, dihydrotestosterone 4.5% and <1% with all other steroids tested (Spectria specifications).

Statistical Analyses

Changes in plasma concentrations of testosterone over 24 hours and after injection of hCG or buserelin were analysed by a single-factor repeated-measures ANOVA, with an antedependence error structure (Wang and Goonewardene, 2004). Analysis was carried out using the MIXED procedure in version 8.2; SAS (Cary, NC). Following the analyses of the individual experiments, Spearman's rank correlation coefficient (Conover, 1999) was used to test for association between the average testosterone concentrations over 24 hours and the maximum observed testosterone concentration in each of the stimulation experiments.

Results

Figure 1 depicts the changes in plasma testosterone concentrations for individual koalas over a 24-hour period. For koala K5, concentrations of testosterone ranged from 0.9 ng/mL (2000 h) to 8.8 ng/mL (1600 h). Two koalas (K1 and K6) had plasma testosterone
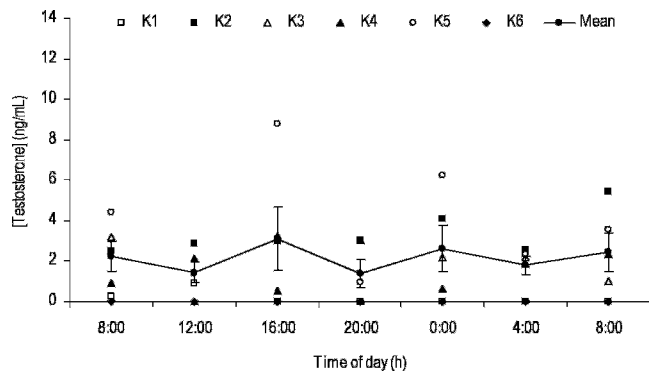
Figure 1. Changes in plasma concentrations of testosterone for individual koalas (K1–K6) during a 24-hour period in early spring. Also shown is the mean ± SEM (n = 6) profile over 24 hours.

concentrations below the detection limit of the assay at the majority of sampling times. Based on the 4-hourly measurements of testosterone concentration used in this study, there was no apparent diurnal rhythm in plasma testosterone concentrations when the data were pooled for the 6 koalas.

Changes in plasma testosterone concentrations of individual koalas after the injection of buserelin and hCG are depicted in Figures 2 and 3, respectively, and the data are summarised in the Table. Koala K6 was not included in the analyses, as this animal did not show testosterone concentrations above the sensitivity of the assay. Both hormones induced an increase ($P < .05$) in plasma testosterone, with maximum concentrations (buserelin, 5.8 ± 2.2 ng/mL; hCG, 7.6 ± 2.9 ng/mL) occurring at 90 and 120 minutes after the injection of buserelin and hCG, respectively. Testosterone tended to decline beyond 90 minutes after treatment with buserelin, but this was not significant. In contrast, maximal concentrations of testosterone were maintained for 240 minutes after the injection of hCG.

The mean change in testosterone concentration ($\Delta[T]$; where $\Delta[T] = [T]_{Tx} - [T]_{T0}$) when calculated over all
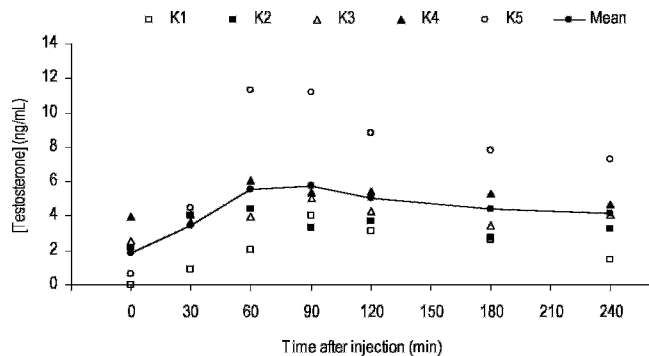

Figure 2. Plasma concentrations of testosterone for individual koalas (K1–K5) after the injection IM of buserelin (4 μg). The sample at time 0 was taken immediately before the injection of buserelin.

time periods (time 30 to 240 minutes) after injection of hCG and GnRH was 4.69 ± 0.52 ng/mL and 2.87 ± 0.53 ng/mL, respectively.

A Spearman's rank correlation revealed a significant association between the average testosterone concentration over 24 hours of all 6 koalas and the maximum observed testosterone concentration in the hCG stimulation experiment ($r = 1.0; P < .01$). There was also a positive correlation between the average testosterone concentration over 24 hours and the maximum observed testosterone concentration following the GnRH stimulation test ($r = .772; P = .07$), but this was not significant at $P < .05$.

Discussion

The findings in the present study demonstrated that plasma concentrations of testosterone in male koalas can show marked fluctuations during a 24-hour period, a finding which is generally consistent with those of studies by Carrick and Cox (1977), Carrick et al (1981), McFarlane (1990), and Cleva et al (1994). There were also large differences between individual koalas in plasma testosterone concentration, and it was not possible to relate these differences to behavior or social position of the koalas. Two koalas that had particularly low testosterone during the 24-hour period were wild sourced (Koala K1) or had been held in captivity (Koala K6). The background of the koalas appeared, therefore, not to be related to plasma concentrations of testosterone.

There was no evidence in this study of a diurnal rhythm in testosterone secretion either for individual koalas or when data were pooled for 6 animals. It is possible that the failure to observe diurnal secretion of testosterone may have been associated with the use of repeated anesthesia and/or a consequence of the
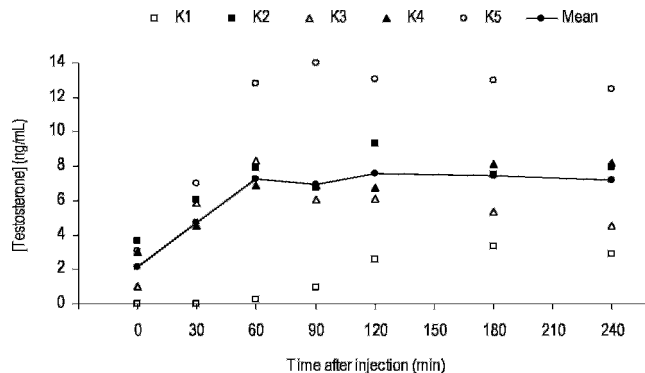

Figure 3. Plasma concentrations of testosterone for individual koalas (K1–K5) after the injection of hCG (1000 IU). The sample at time 0 was taken immediately before the injection of hCG.

Plasma testosterone concentrations (mean ± SEM, n = 5) after the injection of buserelin (4 μg) or hCG (1000 IU) at Time 0*

	Time (min)						
Stimulation test	T0	T30	T60	T90	T120	T180	T240
Buserelin	1.87 ± 0.71[a]	3.43 ± 0.64[b]	5.56 ± 1.57[c]	5.78 ± 1.39[c]	5.07 ± 1.01[c]	4.38 ± 0.98[bc]	4.13 ± 0.97[bc]
hCG	2.16 ± 0.70[a]	4.7 ± 1.24[b]	7.23 ± 2.04[c]	6.92 ± 2.09[c]	7.56 ± 1.74[c]	7.46 ± 1.62[c]	7.21 ± 1.66[c]

* Different superscripts within rows indicate differences between means are significant ($P < .05$).

relatively long interval between blood sampling (4 hours) used in this study. With regard to possible effects of repeated anesthesia, this did not prevent the occurrence of large fluctuations in testosterone which presumably would have been preceded by pulses of LH and therefore GnRH. The random, large fluctuations in koala plasma testosterone concentrations emphasized the importance of a practical stimulation test that would allow the viable and reliable assessment of prevailing testosterone secretory capacity.

Both the GnRH agonist buserelin and hCG induced testosterone secretion in male koalas. The initial phase of the testosterone response curve was similar for buserelin and hCG, and near maximal testosterone concentrations typically occurred around 60 minutes after injection. There were positive relationships between average testosterone secretion during 24 hours and the maximal concentration achieved after injection of buserelin ($P = .07$) and hCG ($P < .01$). For example, Koala K5 had the highest testosterone concentrations during 24 hours and also showed the greatest testosterone response after the injection of both buserelin and hCG. Koalas K1 and K6, on the other hand, had low plasma testosterone during 24 hours, and Koala K1 showed the lowest testosterone responses to both buserelin and hCG, while Koala K6 did not show a response to either exogenous hormone. Koalas K2, K3, and K4 had intermediate concentrations of testosterone during 24 hours and showed intermediate responses to buserelin and hCG. It is concluded from these findings that a GnRH agonist and hCG can be used in a testosterone stimulation test to gain an accurate index of prevailing testosterone secretion for individual male koalas. The failure to induce a testosterone response with natural sequence GnRH in an earlier study in male koalas (McFarlane, 1990) may have been due to an insufficient dose to generate an adequate LH response and/or the rapid clearance of natural sequence GnRH from circulation. The testosterone response to buserelin after 90 minutes in this study may be explained by either continued down-regulation of GnRH receptors or the relatively rapid clearance of buserelin from the circulation.

The testosterone response curves after the injection of buserelin and hCG showed subtle differences. After injection of buserelin, testosterone typically peaked around 60–90 minutes, and this was followed by a decline to 240 min. It is most likely that injection of buserelin induced an acute release of LH from the pituitary, after which the gonadotroph cells became refractory to further stimulation by buserelin. A classical desensitisation of the gonadotrophs occurs in response to both endogenous and exogenous natural sequence GnRH and also GnRH agonists (Conn et al, 1999). Following injection of buserelin, therefore, the Leydig cells would have been exposed to a transient peak of LH similar to that which normally occurs in males. In contrast, after injection of hCG, the Leydig cells would have been exposed to chronic stimulation by hCG, which has a relatively long half-life in circulation (Birken et al, 1999). The response to hCG indicated that the Leydig cells in koalas have the capacity to sustain elevated testosterone secretion when exposed to chronic trophic hormone stimulation.

In summary, the present study has demonstrated that a GnRH agonist and hCG can be used to obtain an accurate index of testosterone biosynthetic capacity in male koalas. A practical testosterone stimulation test would involve the injection of exogenous hormone and collection of a single blood sample between 60 and 90 minutes after injection. The removal of the need for multiple blood samples to determine testosterone status makes the testosterone stimulation test particularly applicable in longitudinal studies of reproductive function in male koalas. This will allow changes in reproductive function including behavior to be more accurately related to changes in testicular endocrine activity. In this regard, a relationship was reported between the testosterone response to GnRH and social status in the sugar glider, which is also a marsupial (Bradley and Stoddart, 1997).

Acknowledgments

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