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ABSTRACT

GnRH treatment has been suggested to increase testosterone levels temporarily and to stimulate libido in stallions, but its use has not fully ascertained in dromedary camels. The aim of this work was to study the effects of administering 100 µg of GnRH on testosterone profile, libido and semen parameters in dromedary camels. The same bulls were used as self-controls and experimental group. Blood samples were collected every 20 minutes (T0-T12) for 4 hours, and semen collections were performed over a 2-hour period after T12. GnRH was administered immediately after T0. In GnRH-treated bulls, testosterone levels showed an upward trend, peaking after 140 minutes, and then slowly decreasing. GnRH administration also led to a decrease in mating time and an increase in spermatozoa concentration. Overall, it seems that administration of 100 µg GnRH might increase testosterone levels temporarily and enhance camel reproduction performance.

Keywords: *Camelus dromedarius*, GnRH, Testosterone, Sexual behavior, Semen Collection, Ejaculates Quality.

1. Introduction

The short breeding season and lack of libido of dromedary bulls are the major complaints in dromedary camel reproduction management. The seasonality of the animals, their libido, and their mating performances are, indeed, complex traits affected by management, environmental, nutritional, psychological, hormonal, and physical factors (Al–Qarawi, 2005; Stout, 2005; Fatnassi et al., 2014a). Depending on those factors, the duration of the breeding season varies from 2 to 6 months (El–Wishy, 1988); for instance, in the United Arab Emirates it lasts from October to late April (Tibary & Anouassi, 1997), while in Tunisia it is reported to be from December to March (Hammadi et al., 2008) or even April (Burgermeister, 1975). The libido of male dromedary camels generally peaks during the coolest, rainy months of the year, and then declines; some males lose libido early, while others maintain it for longer periods. However, the reasons for this variability are still not fully understood (Deen et al., 2003).

High testosterone levels are responsible for morphological and histological changes in the testicles and in the accessory sex glands, as well as for augmentation of the camel bull's libido and typical sexual behavior patterns (Yagil & Etzion, 1980; Tingary et al., 1984; Azouz et al., 1992; Pasha et al., 2013; Fatnassi et al., 2014b). Consequently, it has been reported that, outside the breeding season, the decline in libido is due to testosterone concentration falling below a critical level (Deen et al., 2005; Deen, 2008). Low testosterone concentration could be responsible not only for the failure of male libido, but also for increased reaction times, increased number of mounts per successful ejaculation, lower ejaculate volume and lower number of sperms per ejaculate (Al–Qarawi, 2005). The use of artificial insemination (AI) in dromedary camels may solve the problem of low reproductive performance due to male seasonality (Skidmore et al., 2013). However, mating failures also lead to low semen volume, a decreased concentration in spermatozoa and less ejaculates, which has the additional knock-on effect of reducing the number of frozen semen doses for artificial insemination (El–Hassanein, 2003; El–Bahrawy, Unpublished data).

Different treatments have been proposed to enhance the libido and mating ability of dromedary camel bulls. A testosterone propionate treatment has been used, but this treatment resulted in decreased testicular weight and size, reduction in sperm production and lower sperm motility (Al–Qarawi et al., 2001; El–Belely & Al–Qarawi, 2003). The administration of 100 µg of gonadorelin (GnRH analogue) has been suggested to temporarily increase blood testosterone levels in stallions to stimulate their libido and mating ability (McDonnell, 1992; Stout, 2005). The effects of GnRH have also been previously investigated in dromedary camel bulls, but results are conflicting. It has been reported that continuous administration of 175 µg of gonadorelin induced a clinical improvement in the male camel's libido outside the breeding season (Moslah et al., 1992). However, endocrinological investigations into this therapeutic regimen showed that the high testosterone levels (above 20 ng/ml), reached during the first 4 days of the treatment, induced a negative feed-back on the Hypothalamic-Pituitary-Gonadal axis, resulting in a decrease in the bulls' testicular diameter and in a significant drop in their testosterone levels (Quaranta et al., 2010).

It has been hypothesized that 100 µg of gonadorelin might temporarily raise plasma testosterone levels and libido in camel bulls, so the aim of this work was to characterize plasma testosterone levels for 4 hours after this treatment. Semen collections were also carried out, between 4 and 6 hours after GnRH administration, and mating behaviour and semen parameters were evaluated.

2. Material and Methods

2.1. Animals and housing

The trial was carried out at the end of the breeding season, from the 20th to the 29th March 2013, in camels reared under intensive system at the Arid Lands Institute's experimental station in Médenine, Tunisia (33° 30' N, 10° 40' E). Five clinically healthy male dromedary camels (*Camelus dromedarius*), with a mean body weight of 516 ± 25 kg and good body condition score (3.5 ± 0.3 arbitrary units; range from 0 to 5 (Faye et al., 2001)), were used for this study.

In the previous summer, each bull was kept in a single open-air paddock shaded by trees whereas, starting from October, they were put into single stalls (Height = 3 m, Length = 5 m and Width = 3 m) with sand floors. The stalls were located far from the females' pen, thus preventing any visual or physical contact between the bulls and the dams. Feeding quantity and quality remained constant during the breeding season and throughout the whole experiment. The diet met the maintenance requirements, and water was made available once every two days (Laudadio et al., 2009).

During the breeding season, all bulls were used for semen collection and were normally divided into two groups (Group I: camel 504, 514, 515; Group II: camel 8, 808). They were well accustomed to this practice and to their husbandry system. To avoid any changes to normal management during the trial, the division between the two groups was maintained throughout.

2.2. Experimental design

The experiment lasted 9 days. On day 1, indwelling jugular catheters were inserted once slight sedation of the animals (5 mg/100 Kg b.w. of Acepromazine, Combistress®, Belgium), surgical preparation of the neck, and local anesthesia (Lidocaine 2%, Unimed, Tunisia) had been conducted at the catheter insertion site. The catheters were regularly flushed with heparin solution (20 I.U./ml) throughout the experimental period.

On day 2 and 3, blood and semen samples were collected from Group I and Group II, respectively, in order to obtain baseline testosterone measurements (Self-Control: SC).

On days 4 and 5, blood and semen samples were again collected from Group I and Group II, respectively. This time, 100 µg gonadorelin diacetate tetrahydrate (GnRH analogue) (Fertagyl®, International Intervet) was injected through the catheter immediately after the first blood sample. To increase the number of observations, the experiment was repeated twice more, first on day 6 and 7, and then on day 8 and 9. GnRH was thus administered to each animal a total of three times, once every 48 h, and blood and semen collections were performed after it..

Blood sampling procedure

In the self-control group (SC), blood samples were drawn at 20-minute intervals from 2.00 p.m. (T0) to 6.00 p.m. (T12), for a total of thirteen blood samples. In the experimental group (EG), GnRH was administered immediately after the first blood sample (T0, at 2.00 p.m.); the blood samples were then collected according to the same schedule. Blood samples were collected through the catheters using syringes and then drawn into Venoject® tubes (Terumo Europe, Leuven, Belgium) with lithium heparin, and kept in ice until plasma was separated, within 2 h of collection, by centrifugation at 4°C for 15 min at 3000 rpm. Plasma samples were stored at -20°C until analyzed for testosterone concentration.

Semen collection procedure

The first semen collection session took place within 10 minutes of T12 (the end of serial blood sampling) and the mating order of the males was randomized. Briefly, the first male began the semen collection procedure around 6.00 p.m., while each consecutive male started collection as soon as the previous procedure had been completed. All procedures were carried out over a two-hour period after T12, from 6ish p.m. to 8ish p.m. A receptive female was led into the open paddock, near the males' stalls, and restrained in sternal recumbence. A bovine artificial vagina (IMV, France), with a water-jacketed tube located at the end of the collection funnel, was used for semen collection. When the operators were ready, the door of the male's stall was opened, the mating session began and was recorded by a video camera (Sony Camcorder digital video). The semen collection sessions were scheduled, according to a standard method, proposed by Padalino et al. (2015). Briefly, the semen collection was scheduled according to the following timings: 1) maximal latency time before mounting (the time from the moment the male exited the stall until he sat for the first time on the female for mounting): 15 min. 2) maximal mating time (the time from first sitting on the female to his return to the stall = service/ejaculation time + standing over the female + walking around): 45 min. 3) maximal time between two copulations: 30 minutes. 4) maximal standing on/over the female time (the time when the male camel is near or over the female): 30 min. 5) maximal walking around time (the time when the camel is walking in the semen

collection pen, being uninterested in, and distant from, the female): 6 min. When a camel exceeded one of the above time limits, the session was ended.

2.3 Analysis of parameters

Testosterone analysis

Plasma testosterone concentrations were determined by radioimmunoassay (RIA) (Immunotech Beckman Coulter Company, Ref 1087, Marseille, France), a gamma counter was used for counting, and the resulting number was converted by way of a calibration curve to measure the hormone levels in unknown samples. Sensitivity was 0.04 ng/ml and intra- and interassay coefficients of variation were 7.4% and 11.1%, respectively.

Behavioral parameters

The semen collection videos were analysed by filling out a "focal animal sampling" ethogram (Padalino et al., 2013). The durations of the following behavioral states were noted down: latency time, service/ejaculation time (ST) (time of copulation with the artificial vagina), standing near/over the female (SOF), walking around (W); consequently, total mating time was calculated by adding together ST, SOF and W. Moreover, occurrence of the following behavioral events was also recorded: number of mounts, sound emission, defecation, urination, tail flapping, blathering, *dulaa* extrusion, yawning, teeth grinding, neck touching, sniffing and *flehmen* (Fatnassi et al., 2014b). The frequency of behavioral events was expressed as number of events per minute (n/min). Finally, the male camel libido was scored (Padalino et al., 2013).

Semen parameters

Ejaculates were evaluated within 15 minutes after collection and the following parameters recorded: volume, mass motility, viability, concentration and total sperm number. Volume was calculated by reading directly from the graduated collection tube. Mass motility was evaluated by placing 10-20 µl of undiluted semen on a pre-warmed slide and, after covering it with a coverslip and waiting for 5 minutes, by direct observation under microscope at 200x magnification; all motile sperm, whether oscillatory or progressive, were considered motile and used to generate a value for mass motility: an

arbitrary score from 0 (immotile sperms) to 5 (highly motile) was assigned. The percentage of viable spermatozoa was evaluated using Eosin/Nigrosin staining (Skidmore et al., 2013). The sperm concentration was calculated after complete spontaneous liquefaction of the ejaculates using a haemocytometer (Wani et al., 2008). Total sperm number was calculated by multiplying sperm concentration by volume.

2.4. Statistical analyses

Testosterone data were normally distributed. Normal distribution was checked using the Anderson-Darling test. Testosterone concentration values were subjected to repeated-measures analysis of variance using the Generalized Linear Model procedure (SAS, 1999). The factors were: group (SC, EG), time (T0-T12), repetition (1^{st}, 2^{nd}, 3^{rd}), and the interactions group*time, group*repetition, time*repetition and group*time*repetition. Tukey's post hoc test was used to perform multiple statistical comparisons. In addition, the Dunnett's two-tailed *t* test was used to test the statistical difference between times (T0-T12) in the two groups (SC, E), considering T0 as a control. Data were expressed as mean and mean standard error (SE).

Since the number of observations of behavioral and seminal parameters was low, the latter data were statistically analysed by the Mann-Whitney U test using GenStat 64-bit Release 16.2. The factor was the group (SC, EG). The *p*-level was always set to 0.05. All data were expressed as mean ± standard deviation.

3. Results

3.1 Testosterone concentration

The plasma testosterone profile from T0 to T12 in GnRH-treated (EG) and self-control (SC) dromedary camel bulls is shown in Figure 1. Testosterone concentration was significantly influenced by the following factors: group (df = 1; $F_{(2,12)} = 5.29$; P = 0.03), time (df = 12; $F_{(2,12)}$= 11.88; P < 0.0001), and group*time (df = 12; $F_{(2,12)} = 4.24$; P < 0.001). However, group did not

affect testosterone basal level (T0, SC *vs* E : 3.0 ± 0.8 *vs* 3.5 ± 0.9 ng/ml; P= 0.94) and no effect of repetition was found (P=0.41).

Starting from T1, testosterone levels showed an upward trend only in treated animals (EG), becoming statistically significant after 1 hour (T3, SC *vs* EG: 2.7 ± 0.7 *vs* 8.8 ± 2.6 ng/ml; P=0.02), peaking after 140 minutes (T7, SC *vs* EG: 2.8 ± 1.8 *vs* 11.9 ± 3.8 ng/ml; P=0.007), and then slowly decreasing (T12: SC *vs* EG: 3.6 ± 1.1 *vs* 8.7 ± 1.6 ng/ml; P= 0.02). GnRH, as expected, caused a steady increase in plasma testosterone levels and the maximal values were reached between two and three hours after administration.

3.2 Behavioral data

Table 1 shows in detail the behavioral data recorded during semen collection in both groups. The GnRH-treated bulls showed shorter mating times (EG: 31.64 ± 11.69 vs SC: 44.0 ± 2.0 min; U:7.5, P=0.05), service times (EG:3.54 ± 3.75 vs SC 8.25 ± 4.34 min; U:7., P=0.03), and standing over the female times (EG: 18.45 ± 12.66 vs SC: 31.75 ± 5.37 min; U:3.5, P=0.01). From the limited data available, it seems that GnRH leads to faster semen collection, with a decrease in service and standing over the female times, and overall shorter mating times.

3.3. Sperm quality

Data for sperm quality are presented in Table 2. Ejaculates collected in the treated animals would appear to show an improvement in all the considered parameters. However, only sperm concentration was significantly higher in the ejaculate collected from GnRH-treated bulls (EG: 1085 ± 274.6 vs SC: 491.3 ± 378.1 10^6/ml; U:4, P=0.02).

4. Discussion

In this study, we tested the effects of administering 100 µg of GnRH analogue on testosterone level and preliminarily on mating behaviour and semen quality in dromedary bulls. As hypothesized, our results show that this GnRH treatment significantly increases testosterone levels in camel bulls.

From our preliminary data, it also seems that the treatment has a positive effect on semen collection, reducing the mating time and increasing the sperm concentration.

The testosterone rise pattern observed in our camel bulls during the first 80 minutes after injection (PGI: Post GnRH Injection) is in agreement with previous studies. A similar testosterone trend had previously been observed in GnRH-treated cattle bulls (Devkota et al., 2011) and GnRH-treated stallions (Roser & Hughes, 1992). The testosterone peak observed 2 hours after the gonadorelin injection in the dromedary camel is also similar to the testosterone peak observed after a single administration of GnRH in cattle bulls (Post et al., 1987). The latter authors investigated the effects of different GnRH doses (from 20 μg to 600 μg) on bulls' testosterone levels and found that the testosterone peaking time and levels were not dose-dependent. Consequently, a time window of between 2 to 3 hours PGI was proposed for measuring plasma testosterone levels in GnRH-treated bulls. Our data suggest that, after GnRH injection, testosterone peaked in the same time window in dromedary bulls. Consequently, this timing could be used to detect blood testosterone levels post injection, and to schedule semen collection sessions during the testosterone peak in camels.

In our study, 100 μg of GnRH were administered and testosterone levels monitored until 4 hours post injection. Testosterone concentration started decreasing at 180 minutes after injection and, at the time of the last blood sample, the testosterone concentration in GnRH-treated bulls was still significantly higher than both T0 baseline levels and those of the self-control group. In bulls, Post et al. (1987) proved that the time required for testosterone to return to basal levels, following the 3rd hour PGI, is affected by the dose of GnRH used, whereas testosterone peaking level is unaffected by GnRH dose. Since we tested only 100 μg of gonadorelin and we monitored the testosterone profile only for 4 hours, more investigations are required in order to assess whether the duration and the amplitude of the GnRH-induced testosterone curve are also affected by the GnRH dose in the male dromedary camel.

From our data there was no significant effect of repetition, while testosterone levels at T0 were not significantly different between GnRH-treated and self-control bulls. Thus, it is reasonable to

suppose that such a therapeutic regimen does not affect Hypothalamic-Pituitary-Gonadal axis homeostasis and could be used to increase testosterone levels temporarily at the end of the breeding season. However, a stimulation test with careful determination of pituitary and testicular hormones (LH, FSH, Testosterone, Inhibin) would probably be the gold standard test to confirm this assumption.

The capacity of testes to respond to a GnRH stimulation test is significantly lower in subfertile stallions and bulls (Devkota et al., 2011; Roser & Hughes, 1992). Our results demonstrated that camels responded correctly, as expected, as only fertile healthy bulls were enrolled in the study. Further studies on the effects of a GnRH stimulation test would be of significant importance also in dromedary camel bulls with low libido or poor-quality ejaculates.

GnRH treatment has been suggested to modulate male sexual behavior by increasing circulatory levels of testosterone (McDonnell, 1992) and it is reported that, in rat males, an induced increase in testosterone levels almost immediately shortens the latency to mount (James & Nyby, 2002). Furthermore, it has also been shown that, following testosterone injection, penile reflexes can be restored in a very short time (6 min) in long-term castrated rats (Sachs & Leipheimer 1988). Testosterone acts by improving the expression of sexual motivation and by coordinating genital reflexes and the somatomotor patterns of copulation, and these effects could be elicited by slow-acting genomic effects, quick genomic effect and non-genomic fast acting mechanisms (James & Nyby, 2002; Hull et al., 1999). In our study, there was no decrease in latency time, as reported in rats, but an overall decrease in mating time. Service time and time spent over the female dropped significantly, and these variations could have been caused by the higher level of testosterone. The reduction in mating time and consequently of the semen collection procedure could be useful in dromedary camel breeding management, because camels are slow during mating and lengthy semen collection is considered wearisome for the operator (Padalino et al., 2015). However, any positive effect of GnRH on mating behaviour should be ascertained in future studies, since in this

preliminary study we recorded a low number of semen collections which started four hours after GnRH treatment, i.e. during the decreasing testosterone phase.

The semen parameters recorded generally resemble normal ranges and average values (Deen, 2008; Wani et al., 2008; Tibary & Anouassi, 1997; Hammadi et al., 2008); lower ejaculate volume and higher sperm concentration were reported after GnRH injection (EG) compared with the self-control (SC). This data could be explained by the reduced service time, which may have contributed to the significant difference in mean sperm concentration, without variation in total sperm number.

Our study was limited by the small number of animals recruited, by the low number of ejaculates collected (one before and three after GnRh for each animal), and also by the wrong time window for performing semen collection. Therefore, the effects of GnRH injection on dromedary bull seminal parameters and libido presented herein are preliminary and need to be verified by collecting a large number of ejaculates two hours after treatment, during the GnRH-induced testosterone surge.

Conclusion

Overall, a 100 µg dose of GnRH may lead to a temporary increase in testosterone levels, a decrease in mating time, and a rise in sperm concentration in male dromedary camels. From our preliminary results, it appears that the treatment might increase testosterone levels and improve the semen collection procedure in dromedary camel bulls at the end of the breeding season. However, further studies will be required to ascertain the effects of GnRH-induced testosterone levels on mating behavior and seminal parameters, conducting semen collection between 2 and 3 hours after GnRH administration, when testosterone peaks.

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Statement of Animal Rights

All the experimental procedures were conducted in conformity with the European Union Directive for the protection of experimental animals (2010/63/EU). All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Authorship:

BP, DM, MH and GML conceived the work. BP, DM and MF carried out the experimental parts of the trial. LA recorded behavioral data and analysed the video, MF and KT performed the hormonal analysis. BP, MH and TK analysed the data. BP, DM, MH and GML wrote and revised the manuscript. All authors read and approved the final manuscript.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the manuscript.

Statement

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Table 1. Mating behavioral data recorded in dromedary camels four hours after the injection of GnRH (EG) (Fertagyl®,100 µg) and in the same animals without treatment (SC). Data are expressed as mean ± standard deviation.

Table 2. Dromedary camel sperm quality of ejaculates collected four hours after the injection of GnRH (E) (Fertagyl®, 100 µg) and in the same animals without treatment (SC). Data are expressed as mean ± standard deviation

Fig. 1. Plasma testosterone profile of dromedary camel bulls from T0 (2 p.m.) to T12 (6 p.m.) in the experimental group (E) (after the i.v. administration of 100 µg Fertagyl®,) and in the same animals without treatment (Control)

$^{A, B}$ significantly differs ($P < 0.05$); * different from T0 ($P < 0.05$); ** different from T0 ($P < 0.01$).

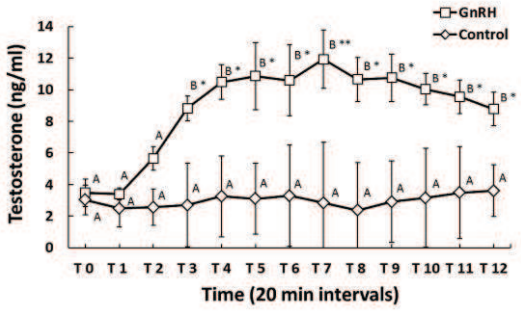

Table 1. Mating behavioral data recorded in dromedary camels four hours after the injection of GnRH (EG) (Fertagyl®,100 µg) and in the same animals without treatment (SC). Data are expressed as mean ± standard deviation.

Behavior	EG (GnRH)	SC (Control)	P value
Latency time (sec)	67.6 ± 15.6	64.0 ± 12.2	0.628
Service time (min)	3.5 ± 3.7	8.2 ± 4.3	0.037
Standing over female (min)	18.4 ± 12.6	31.7 ± 5.4	0.012
Walking (min)	9.4 ±6.1	4.0 ± 5.3	0.057
Mating time (min)	31.6 ± 11.7	44.0 ± 2.0	0.050
Blathering (n/min)	0.9 ± 0.6	0.6 ± 0.5	0.661
Dulaa extrusion (n/min)	0.09 ± 0.2	0.15 ±0.3	0.967
Flehmen (n/min)	0.32 ± 0.25	0.15 ± 0.26	0.365
Number of mounts	3.80 ± 0.2	4.10 ± 0.4	0.851
Neck touching (n/min)	0.33 ± 0.5	0.10 ± 0.20	0.755
Sniffing (n/min)	1.78 ± 1.12	0.56 ± 0.66	0.026
Sound Emission (n/min)	0.08 ±0.12	0.01 ±0.03	0.554
Tail flapping (n/min)	1.76 ± 2.5	1.15 ± 2.7	0.800
Libido Score	3.2 ± 0.8	3.0 ± 0.4	0.142

Table 2. Dromedary camel sperm quality of ejaculates collected four hours after the injection of GnRH (E) (Fertagyl®, 100 μg) and in the same animals without treatment (SC). Data are expressed as mean ± standard deviation

Parameter	E (GnRH)	SC (Control)	P value
Volume (ml)	4.8 ± 3.4	7.3 ± 4.8	0.341
Motility score (0-5)	2.4 ± 1.3	1.2 ± 1.6	0.230
Viability (%)	66.3 ± 9.7	54.1 ± 22.1	0.548
Concentration (10^6/ml)	1085.1 ± 274.6	491.2 ± 378.1	0.022
Total Sperm Number (10^6/ml)	5141.0 ± 3535	3043.4 ± 2758	0.326

Highlights

- GnRH analogue (100 μg of gonadorelin) was administered to male dromedary camels
- In GnRH-treated bulls, testosterone shows an upward trend, peaking after two hours
- GnRH leads to a decrease in mating time and an increase in semen concentration
- GnRH may be used as "short therapy" to enhance camel reproduction performance