Unique strategies to control reproduction in camels

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The reproductive efficiency of camels is low under natural pastural conditions and so the use of artifical insemination and embryo transfer are becoming increasingly important to improve their breeding potential. Methods to control their reproductive cycle are therefore essential. This review describes characteristics of the ovarian follicular wave pattern in camels and exogenous hormonal control of ovulation. It also summarizes the difficulties involved with artifical insemination and analyzing the highly gelatinous semen, and reports on the latest methods used to try and reduce the viscosity and liquefy camel semen. In addition an account is given of different hormonal and physical methods used to synchronise follicular waves, and various hormone treatments used to broaden the availability of ovulated, asynchronous and non-ovulated recipients are discussed.

Introduction

The family Camelidae originated in North America and split into three genera, *Camelus, Lama* and *Vicuña* approximately 11 million years ago (Stanley *et al.* 1994). Today there remains two species of large Old World Camelids indigenous to Africa and Asia namely, *Camelus bactrianus* (the Bactrian or two-humped camel) and *Camelus dromedarius* (the dromedary or one humped camel) and four species of New World Camelids, the domesticated llama (*Lama glama*) and alpaca (*Vicugna pacos*), and the wild guanaco (*Lama guanacoe*) and vicuña (*Vicugna vicugna*).

Both dromedary and Bactrian camels exhibit some unique aspects of reproductive physiology compared with other large domestic animals. For example, camels have a short breeding season during the cooler winter months (Wilson 1984), induced ovulation in response to coitus (Marie & Anouassi 1986), a very slow rise in peripheral serum progesterone concentrations after ovulation and a short luteal phase of only 8 – 10 days in the non – pregnant animal (Marie & Anouassi 1987; Skidmore *et al.* 1995) and a long gestation period of 13 months (Mehta *et al.* 1962). In addition they exhibit a long (8 – 10 month) period of lactation-related anoestrus which leads to a long inter-calving interval (Nawito *et al.* 1967). This low reproductive efficiency could be increased by various strategies to control the reproductive cycle and increased use of assisted reproduction techniques such as artificial insemination and embryo transfer. This paper briefly describes ovarian follicular dynamics in camels and outlines methods that can be used to control their reproductive cycle and increase breeding efficiency.

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Ovarian follicular dynamics

All camelids are induced ovulators. Therefore, during the breeding season follicles pass through periods of growth, maturity and regression if ovulation is not induced by mating (Musa & Abusineina 1978; El Wishy 1987). The use of serial real-time ultrasonographic examinations has shown that although the follicular wave pattern varies considerably between camels it can be divided into 3 phases: i) the growth phase which lasts 6 – 10 (±0.5) days; ii) a mature phase of approximately 7 – 8 (± 0.8) days, and (iii) a regression phase of 11 –12 (± 0.8) days (Skidmore *et al.* 1996). In all instances the new follicles become visible and start to grow before the mature follicle has completely regressed to give an interwave interval of 18.2 (± 1.0) days in dromedary camels (Skidmore *et al.* 1996) and 19 days in Bactrian camels (Niasari-Naslaji 2008).

In approximately 50% of follicular waves exhibited by domesticated female camels left separate from male camels the dominant follicle reaches a mature size of 1.3 –1.9 cm diameter, at which time it still responds to an ovulatory stimulus. In the remaining 50% of follicular waves however, the dominant follicle continues to grow to as large as 3.0 – 6.5 cm diameter when it will not ovulate. These large follicles take as long as 18.4 ± 0.8 days (range = 11–33 days) to reach their maximum diameter, they remain at the same size for 4.6 ± 0.5 days and take 15.3 ± 1.1 days to regress (Skidmore *et al.* 1996), although others have reported they can take anything from 8 – 45 days to regress (Tibary & Anouassi 1996). The speed of regression depends on the morphological characteristics of the anovulatory follicle as some follicles have a thin and richly – vascularized wall whilst others have an opaque and thick wall. In addition the contents are usually serous in the early stages but soon become haemorrhagic and show clotted blood and organized fibrin in the later stages (El Wishy 1988; Tibary & Anouassi 1997). Clearly, these overlarge follicles derive from the modern management practice of keeping male and female camels apart during the breeding season but the hormonal mechanisms that drive the development of such structures, and the reasons why they occur in only a proportion of unmated camels, have yet to be elucidated. As their occurrence can cause significant wastage in embryo transfer or artificial insemination programmes various methods have been applied to try and hasten the regression, or prevent development of these overlarge non-ovulated follicles. For example, Skidmore (1994) treated camels with a post-mature follicle of >3.0 cm diameter with either a single injection of 20 μg buserelin (Receptal) or daily injections of 150 mg of progesterone-in-oil for 14 days. Whereas it took 22 (± 1.5) days for the large follicle to regress in the untreated control group, regression occurred in 14.6 (± 1.3) days in animals injected with buserelin and 12.7 (± 1.5) days in those that received daily injections of progesterone. It is possible that the progesterone therapy suppressed the basal secretion rate of LH from the pituitary gland, thereby preventing any further growth or maintenance of the follicle. However since waiting for overlarge follicles to regress is very time consuming when trying to synchronize groups of donor and recipient animals for embryo transfer, it is preferable to prevent the occurrence of oversized, non-ovulatory follicles by inducing ovulation with an injection of GnRH when the follicle reaches 1.3 – 1.7cm diameter.

Control of ovulation

Previous studies have shown that ovulation can be induced in camelids by mating to an intact or vasectomized male (Marie & Anouassi 1987) or by a single intramuscular (i.m.) injection of seminal plasma (Pan *et al.* 1992; Adams *et al.* 2005). Ovulation must be controlled and synchronized when preparing animals for embryo transfer and artificial insemination. Mating to a vasectomized male, however, or inseminating or injecting seminal plasma is impractical

due both to the difficulty of collecting camel semen and the risk of spreading veneral disease. Therefore, treatment with an LH-like gonadotrophic hormone preparation or a GnRH analogue at the optimal time in the follicular growth cycle is the most practical alternative. Ovulation rates of 80-85 % can be achieved by injecting either 20 μg of the GnRH analogue, buserelin, or 3000 i.u. human Chorionic Gonadotropin (hCG) when the dominant follicle measures 1.0 – 1.9 cm in diameter. This ovulation rate is reduced to < 20% if the follicle measures between 2.0 – 2.9 cm at the time of treatment and to zero if it measures > 3.0 cm (Skidmore *et al.* 1996).

Synchronisation of follicular waves

Previous embryo transfer studies in camels have indicated that optimal pregnancy rates are achieved when the degree of synchrony between donor and recipient camels is 0 – 2 days (McKinnon *et al.* 1994; Skidmore *et al.* 2002). Synchronisation of donor and recipient camels is best achieved either by selecting recipients from a random group of cycling animals or by treating them with a combination of progesterone-in-oil and eCG. Random selection involves serial ultrasonographic examination of the ovaries and administration of GnRH to all females presenting a mature "ovulable" follicle 24 h after the donor is mated (Skidmore *et al.* 2002). This method works well but it is labour intensive and is only feasible when a large number of recipient camels are available. McKinnon *et al.* (1994) synchronised groups of recipient and donor camels by treating them with progesterone-in-oil (100 mg/day) for 10 – 15 days followed by a single administration of 1500 i.u. eCG. Progesterone treatment stopped on the day of treatment with eCG in the donor camels and the recipients received a single dose of eCG 24 h later. This treatment of the recipients with eCG was given to ensure the presence of a mature follicle 24 – 48 h after the donor. However, although the progesterone treatment reduced the rate of follicular growth it did not inhibit it completely, so response to the eCG and time taken for the next follicle to reach a mature size was variable. This method involved daily handling and injection of the camels so it was time consuming, impractical and expensive. More recently Skidmore *et al.* (2009) compared the efficacy of various treatments intended to synchronise follicle wave cycles in dromedary camels by removing the existing follicle of unknown size, by either physical or hormonal means, and replacing it with a follicle capable of ovulating at a known time interval after treatment . Camels were randomly assigned to one of 5 groups and treated with i) 5mg oestradiol benzoate (i.m.) and 100mg progesterone; ii) 20μg GnRH analogue, (buserelin) i.m., iii) 20μg buserelin i.m. on day 0 and 500 μg prostaglandin analogue (estrumate; PG) on day 7, iv) all follicles $\geq$0.5cm were ablated using transvaginal guided ultrasound or v) 5 ml of saline given i.m. (controls). All the camels were subsequently injected with 20μg buserelin 14 days after the first treatment was given (i.e. on day T + 14), and the intervals from treatment to new follicular wave emergence and the day on which the new dominant follicle reached 1.3cm were recorded. The mean interval from treatment to the time taken for new follicular wave emergence and for the new dominant follicle to reach a diameter of 1.3cm was shortest in the ablation group and longest in the oestradiol/progesterone treated group whereas the GnRH and GnRH/PG groups were intermediate (see Table 1). In both the GnRH and the GnRH/PG groups the majority of camels (11/15 in each group) had dominant follicles of 1.3 – 1.9cm in diameter by 14 days after the start of treatment and 21 of the 22 ovulated after GnRH injection given on day T + 14. The ablation, oestradiol/progesterone and control groups however, showed greater variability in follicle size so that fewer of them ovulated after the GnRH injection. The results allowed the conclusion that two GnRH injections given 14 days apart or two GnRH injections 14 days apart plus PG given 7 days after the first GnRH

treatment were the most effective methods to synchronise ovulation in dromedary camels at a fixed interval of 14 days after treatment. Similar results were obtained by Nikjou *et al.* (2008) in their attempts to synchronise follicular wave emergence in Bactrian camel. They compared treating one group of camels with two consecutive treatments of three norgestomet implants and 200mg progesterone i.m. given 7 days apart, with another group of camels that received two injections of GnRH given 14 days apart. The Bactrian camels treated with norgestamet implants did not respond consistently to progestogen treatment and therefore wave emergence was not synchronised, whereas in the group of camels that received two GnRH injections 14 days apart, four of five animals ovulated after the second GnRH indicating that this method was more successful at synchronising follicle wave emergence in Bactrian camels.

Table 1. Mean (±sem) time intervals (days) for new follicular wave emergence and the day on which the dominant follicle reached ≥1.3cm in diameter after oestradiol – 17β + progesterone, GnRH, GnRH + prostaglandin or follicle ablation treatments for the purposes of ovarian synchronization in camels.

	Treatment received				
	E/P	GnRH	GnRH/PG	ABL	Saline
Follicle emergence	6.36 ± 0.83^{a}	3.00 ± 0.48	4.47 ± 0.47	2.33 ± 0.48^{b}	4.3 ± 0.72^{b}
Follicle dominance	12.22 ± 1.01	11.14 ± 0.83	10.73 ± 0.72	8.83 ± 1.08^{c}	12.50 ± 0.57^{d}

E/P 5mg oestradiol benzoate and 100mg progesterone im.
GnRH 20 μg buserelin i.v.
GnRH/PG 20 μg buserelin i.v on T+0 + 500μg prostaglandin (cloprostenol) i.m. on T+7.
ABL follicle ablation
Saline 5ml saline i.m.
Those with different superscripts within rows are significantly different $^{ab}p<0.001$; $^{cd}p=0.044$
(From Skidmore *et al.* 2009)

Methods to broaden recipient availability for embryo transfer

To date embryo transfer experiments in camels have reported pregnancy rates of 60 – 70% when transferring Day 7 embryos to recipients that are negatively synchronized with the donor by 1 – 2 days (McKinnon *et al.*1994; Skidmore *et al.* 2002). However, as discussed above it can be difficult and time consuming to accurately synchronise donors and recipients. It would therefore be of great value to be able to establish pregnancies using non-ovulated or non-synchronised camels as recipients. A number of experimental approaches have been tested.

Treatment of recipients with Progesterone and eCG

Skidmore *et al.* (1992, 2002) showed it is possible to achieve and maintain pregnancies in non-ovulated progesterone-treated recipients by giving them daily i.m. injections of 150 mg of progesterone-in–oil starting 2 days before embryo transfer. This suggested that the degree of synchrony between embryo age and that of the recipient's uterus is perhaps not so important so long as serum progesterone concentrations remain elevated. However, since no CL was present in the ovaries of the recipient camels they required daily injections for the entire 13 month gestation period. This is because the placenta does not contribute to progesterone secretion, and all camelids depend entirely on progesterone from the CL to maintain their pregnancy. As daily injections are not practical for large numbers of recipients, a small number of recipients were each injected subcutaneously with Norgestamet (progestagen) implants at 10 day intervals. However 2 of 4 such treated recipients aborted 10 – 12 days after the start of treatment with the implants, so use of such implants is not recommended (Skidmore *et al.* 1992).

As daily injections are not practical another study investigated the possibility of stimulating the development of follicles in non-ovulated, progesterone-treated pregnant camels by injecting them i.m. with 2000 i.u. eCG on day 25 of gestation, and then inducing the follicles that subsequently mature to 1.3 cm diameter to ovulate with GnRH approximately 10 days later. The CL's that develop maintain the pregnancy for the remainder of gestation. Fourteen of 18 (77%) recipients that had received daily i.m. injections of 75 mg of progesterone-in-oil from 3 days before embryo transfer became pregnant and 7 (50%) remained pregnant after injections of eCG and GnRH. These results show that follicles can develop and subsequently ovulate in progesterone-treated animals and that the fetal maternal recognition of pregnancy signal produced by the conceptus can maintain the CLs that develop, thus eliminating the need for continuous exogenous progesterone therapy throughout pregnancy (J. Skidmore, unpublished data).

A further study investigated the possibility of establishing pregnancies in ovulated asynchronous, progesterone-treated animals. Embryos were transferred on Day 3 or 4 after ovulation into recipients receiving a daily i.m. injection of 75 mg progesterone-in-oil from 2 days before embryo transfer to 6 days after ovulation when it is reduced to 50 mg (day 7) and 25 mg (days 8 and 9). Nine of 16 (56%) recipients became pregnant (ov + 3 n = 4; ov + 4 n = 5) compared with 0/8 controls where the embryos were transferred into non-progesterone treated recipients on Day 4 after ovulation. These results again indicate that the degree of synchrony between embryo age and that of the recipient's uterus is perhaps less important so long as there is a sufficient level of progesterone in the blood. Once more this relieves the need for tight synchrony between the donor and recipient as recipients that ovulate 2 – 3 days after the donor can be maintained on progesterone until the embryo is established and secretes sufficient "maternal recognition of pregnancy signal" to maintain the CL itself (J. Skidmore, unpublished data)

Meclofenamic acid (Arquel)

A previous study has indicated firm evidence for the involvement of prostaglandins in luteolysis in camels as the oral administration of the prostaglandin synthetase inhibitor, meclofenamic acid, prevented both the luteolytic action of exogenous PGF2α and the normal increase in periphereal plasma PGFM concentrations in late dioestrus, thereby prolonging the luteal phase (Skidmore *et al.* 1998). A further study then investigated whether camels treated with meclofenamic acid during the luteal phase could be used as asynchronous recipients for embryo transfer. Meclofenamic acid was administered orally to camels from Day 7 after ovulation until 7 days after embryo transfer, and embryos transferred into these treated recipients on Days 8, 10, or 12 after ovulation. Pregnancy rates of 80%, 60%, or 70%, respectively, were achieved as compared to 10% in the control animals where embryos were transferred into non-treated recipients on Day 8 after ovulation (Skidmore & Billah 2005). This treatment again reduces the need for tight synchrony between donors and recipients as recipients that ovulate 4–5 days before the donor could be maintained on meclofenamic acid until the donor is flushed. This method has the added advantage that the CL is maintained by the conceptus once it is established, and further daily administration of exogenous progesterone or progestagens throughout gestation is unnecessary.

Artificial insemination

AI is an important technique in several species not only to ensure rapid genetic progress but also to enable more efficient use of superior males, eliminate need of transportation of live

animals and reduce the spread of venereal diseases. Working with camel semen however produces many challenges due mainly to the difficulty of collecting and subsequent analysis and handling of the semen.

The preferred method for collection of camel semen is with an artificial vagina but not all males will accept one. It is easier to train young males to use an AV but mating times are not as long as with natural mating and therefore ejaculates may not be complete. Camel semen has a very viscous consistency immediately after collection and as the spermatozoa are entrapped in this viscous seminal plasma they do not display forward progressive motility. This makes spermatozoal motility very difficult to assess and highly variable. It has been reported that semen will liquefy if left on the lab bench for 20 – 30 min, although this does vary between ejaculates (Deen *et al.* 2003). Various other mechanical and enzymatic methods have therefore also been evaluated in an attempt to fully liquefy camel semen. Mechanical methods include gentle pipetting, vortexing, centrifugation and density gradient centrifugation. Only gentle pipetting of semen in a diluent, however, was effective in reducing semen viscosity without compromising sperm motility or viability (Morton *et al.* 2008), but it still does not completely liquefy the semen. There has been comparatively little research on the liquefaction of camel semen with enzymes although Deen *et al.* (2003) examined the effects of α-chymotrypsin (1% α-chymotrypsin in Tris buffer) and caffeine (0.2 mM caffeine supplemented in Tris extender) on spermatozoal motility and found that the addition of caffeine but not of α-chymotrypsin improved motility of individual sperm. In another study, treatment of camel semen with 0.05 mg/mL papain was successful in liquefying semen without detrimental effects to sperm acrosomal membranes (K. Morton, unpublished data). Moreover, the fertility after AI of fresh papain-treated sperm (30 % pregnancy rate) did not differ to fresh non-treated sperm (30 % pregnancy rate; K. Morton, unpublished data) demonstrating the beneficial nature of papain treatment to liquefy camel semen. A number of extenders have been used for fresh and liquid storage of semen but the best results to date have been achieved when the semen is diluted in either i) Green Buffer (I.M.V. Technologies, L'Aigle, France) ® plus 20% egg yolk (v:v; 50% pregnancy rate; Bravo *et al.* 2000; Skidmore & Billah, 2006) ii) an extender containing 11% lactose and 20% egg yolk (v:v; 50% pregnancy rate; Anouassi *et al.* 1992) or iii) INRA – 96 plus (I.M.V.; 36% pregnancy rate; Morton *et al.* 2010).

Artificial insemination also requires induction of ovulation in camels and because ovulation occurs 28 – 36 h after GnRH injection the optimum time for insemination would be 24 h after treatment. Initial studies have shown that pregnancy rates of 50% can be achieved when 300 x 10^6 motile spermatozoa are inseminated (Bravo *et al.* 2000) although more recently insemination of 150 x 10^6 live spermatozoa into the uterine body or just 80 x 10^6 into the tip of the uterine horn ipsilateral to the ovary containing the dominant follicle have both yielded pregnancy rates of 40 – 50% (Skidmore & Billah, 2006). Diluted semen can also be stored in a refrigerator or Equitainer (Hamilton Thorn) at 4°C for 24 h and providing at least 35 – 40% are motile after 24 h it can be inseminated (Bravo *et al.* 2000). However pregnancy rates are dramatically reduced to 20% in camels inseminated with cooled semen diluted in INRA compared with 0% when semen was diluted and cooled in Green Buffer (Morton *et al.* 2010) although previous studies by Bravo *et al.* (2000) did report pregnancy rates of 25% when inseminating semen cooled in Green Buffer for 24 h. Further studies have been carried out by Niasari-Naslaji *et al.* (2005) comparing the use of Green Buffer with their novel extender, SHOTOR diluent (2.6 g Tris, 1.35 g citric acid, 1.2 g glucose and 0.9 g fructose in 100 ml of water) for preserving Bactrian camel semen and it was concluded that the SHOTOR diluent was the better extender for chilling Bactrian semen for up to 12 h but no fertility results were recorded.

Conclusions

The increasing necessity to improve camel production has led to a more scientific approach to management of these animals. Ovulation can be controlled by using GnRH or gonadotrophic hormones if administered at the correct stage of the cycle and pregnancies can be achieved if camels are inseminated with semen diluted in Green Buffer, an extender containing 11 % lactose or INRA – 96 24 h after GnRH injection. The gelatinous nature of the semen makes it difficult to handle but the use of gentle pipetting or treatment with caffeine or papain has been successful in liquefying the semen and thus improves spermatozoal motility.

Hormonal methods using GnRH or a combination of GnRH and PG will successfully synchronise follicular waves in camels which has always been considered a necessary pre-requisite for embryo transfer. It is also possible, however, to achieve pregnancies in non-ovulated progesterone-treated recipients and to induce follicle growth and subsequent ovulation if they are treated with eCG and GnRH. The CLs that develop secrete sufficient progesterone to eliminate the need for continuous exogenous progesterone therapy throughout pregnancy. In addition, recipient availability has been broadened by treatment of ovulated, asynchronous recipients with progesterone or meclofenamic acid so that pregnancies can be successfully achieved in recipients that have ovulated up to 5 days before or 4 days after the donor.

These results show that controlled breeding and strategic use of hormone treatments should increase the efficiency of embryo transfer and AI programmes in camels and therefore improve their reproductive potential.

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