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EFFECT OF ALUMINIUM HYDROXIDE AS A CONJUGATE TO FSH FOR USE IN SUPER-STIMULATION OF OVARIAN FOLLICLES IN DROMEDARY CAMEL (*Camelus dromedarius*)

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ABSTRACT

This study was conducted to develop a simple protocol for super-stimulation of ovarian follicles in dromedary camels using a single subcutaneous injection of commercial FSH product diluted in slow-release diluent, aluminium hydroxide and the feasibility of storing it after dilution at room and refrigeration (4°C) temperatures. In Experiment 1, a total of 50 donors were used, which were divided in 5 groups. The first group was prepared as per our standard protocol with a single injection of 2500 IU eCG and 400 mg FSH (Foltropin-V) in decreasing doses twice daily for 4 days (Control). The second group donors were administered 2500 IU eCG and a single subcutaneous injection of FSH (400 mg) dissolved in 10 mL of aluminium hydroxide. The third group of donors received half the dose of FSH (200 mg) dissolved in aluminium hydroxide in addition to eCG. Groups four and five were administered FSH like group 2 and 3 without eCG. In Experiment 2, FSH diluted in aluminium hydroxide was stored for 14 days at room (Group 2) or refrigeration (Group 3) temperature before administering (200 mg each) subcutaneously to donors as a single injection, in addition to an initial injection of 2000 IU of eCG. Our results show that FSH diluted in slow-release aluminium hydroxide could be used for super stimulation of ovarian follicles in dromedary camels and gives similar results to that of the control group. We have been able to reduce the dose of FSH and its frequency of administration without affecting the quantity of super-stimulation and embryo production. Storing the FSH diluted in Aluminium hydroxide at room or refrigerator temperatures maintains its efficacy up to 14 days and produces similar results to the control group for super-stimulation and embryo production.

Key words: Aluminium hydroxide, dromedary camel, FSH, superovulation

Dromedary camels are fundamental livestock resource providing milk, meat, and draught power in dry regions of Asia and Africa in addition to camel racing, which is a highly sought after and well-organised multimillion-dollar sport in Middle East. Also, camel festivals, including camel beauty contests, and a massive demand for camel milk, leading to establishment of highly mechanised camel dairy farms in different countries, has led to an interest in camel breeding and research, including using multiple ovulation and embryo transfer (MOET) (Wani, 2021). Different ovarian superovulation protocols that are used in other ruminant species have been applied in camels as well, which include use of eCG, (Tinson *et al,* 2001), FSH of porcine (McKinnon *et al,* 1994) or sheep origin (Anouassi and Tibary, 2013), or combination of eCG and FSH (Skidmore and Billah, 2005). The super-stimulation treatment usually starts on Day 4 after induced ovulation of a dominant follicle by an exogenous administration of GnRH (Nikjou *et al,* 2008) or even after two days following injection of GnRH (Ararooti *et al,* 2017; 2018).

The eCG is a complicated glycoprotein having long half-life (40 h) with both LH and FSH activity that represents an advantage because a single dose will induce super stimulation of the ovaries. However, the eCG injection leads to development of 2 variable follicle generations, premature follicle luteinisation, failure of ovulation and big anovulatory follicle development (Anouassi and Tibary, 2013). In contrast to eCG, the half-life of pituitary derived FSH is 5 h and needs frequent injections for inducing super-Stimulation. Two times per day injection of FSH has been found to be more efficient than one time each day, making it labour intensive. In addition, it causes stress to the animal, resulting in a reduced super stimulation

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response (Vyas *et al,* 2004). After subcutaneous injection of follitropin-V diluted in aluminium hydroxide, the concentration of FSH increases gradually in the blood and reaches its peak at 8-12 hours and is still detectable after 96 hours (Kimura, 2016). Keeping in view the above facts that either FSH or eCG or a combination of both has its own problems, the present study was designed to develop a simple protocol for super-stimulation of ovarian follicles in dromedary camels, using a single subcutaneous injection of commercial FSH diluted in a drawback problems slow release diluent aluminium hydroxide.

Materials and Methods

All procedures performed were reviewed and approved by Animal Ethic Committee, in accordance with the regulations of the Ministry of Climate Change and Environment, the government of United Arab Emirates (Permit Number 550353).

Animals

This study was conducted from October 2021 to March 2023. All experiments were performed on the animals maintained at reproductive biotechnology centre Dubai, UAE. Only camels with healthy reproductive tract as examined by ultrasonography with good body condition score (BCC) were selected. Each animal was provided mixed dried hay, water and lick blocks of mineralised salt *ad lib*. In addition, they were provided with 2 kilograms of formulated camel feed every day. Twenty bulls (9 to 15 years in age), with normal fertility were used for natural breeding.

Pre-treatment procedures

All donors were subjected to a protocol for the follicular synchronisation. Briefly, they were injected with GnRH ((100µg, FERTILIN, VETEQUINOL, France) on day-22 and day-12 and intramuscular injections of PGF2α (500µg, BIOESRRUVET VETEQUINO, France) on the day-15 and day-5. A dominant follicle (diameter of 11-17 mm) was expected to be present on either of the ovaries on day 0. All the animals were scanned on day 0 to detect the position and size of dominant follicle (DF) and were treated with GnRH to induce ovulation and synchronise emergence of new follicular wave (Fig 1, A). All donors were examined after 48 hrs to verify the ovulation and only ovulated animal was used in this study for super stimulation.

Experimental design

Experiment 1 was carried out to develop a simple, cost and labour affective protocol for super stimulation of ovarian follicles in dromedary camels. The donor camels (n = 50) aged 8-16 years were used in this experiment. They were randomly divided into five groups with 10 animals in each group. Group 1 of animals received 2500 IU eCG and 400 mg FSH (Folltropin-V) dissolved in saline (20 ml), two times a day intramuscularly (i.m.) for four days in decreasing dose (traditional regimen). Group 2 received 2500 IU eCG and a single injection of 400 mg FSH (Folltropin V), S/C, dissolved in 10 ml aluminium hydroxide (Imject alum, Thermofisher scientific). Group 3 received 2500 eCG and a single injection of FSH 200 mg dissolved in Aluminium hydroxide s/c. Groups 4 and 5 received only 400 and 200 mg FSH diluted in 10 ml inject as a single injection subcutaneously (s/c) without any eCG, respectively.

Experiment 2 was aimed to study the effect of storing diluted FSH in aluminium hydroxide on its efficacy. Thirty donors were divided into 3 groups of 10 animals each. Group 1 was injected with FSH 400 mg in traditional regimen, similar to that of experiment 1 to act as control. Group 2 and 3 received a reduced dose of FSH 200 mg diluted in aluminium hydroxide stored at room and refrigeration temperature for 14 days as s/c injections, respectively. All animals in all the 3 groups also received 2500 IU eCG at the day of starting treatment.

Embryo collection and grading

All donors were scanned to record the number of follicles before mating and number of ovulations 48 h after mating (Fig 1, B). Collection of embryos was performed 8 days after mating in all experiments. The donors were secured in stand-up position in a suitable place and sedated by intravenous injection of 60 mg xylazine. Faeces were removed from rectum and the perineum was cleaned with mild disinfectant. Then the two-way Foley catheter with stylet was guided through the vagina with a sterile hand, then manually the cervix was opened and the catheter was entered. Then the catheter was entered into the internal os of the cervix and the catheter cuff was inflated with 20 to 30 mL of air. Both horns were flushed same time by trans-cervical uterine lavage repeatedly. A total of 500-1000 mL of the flushing medium was used for flushing of every animal. The collected media was filtrated using EmCon filter until 20 to 30 mL of medium remained which was poured into sterile Petri dish and examined under stereomicroscope for embryos and unfertilised ova. Embryos were graded from I-IV depending on the size, macroscopic morphology and stage of development (Manjunatha

et al, 2019). All transferable embryos (Fig 1, C) were transferred to left uterine horn of synchronised recipients.

Statistical analysis

One-way analysis of variance (ANOVA) was used to compare groups. First, the normal distribution of data and homoscedasticity (ANOVA assumptions) were checked using Shapiro-Wilk and Levene's tests, respectively. Then, multiple comparisons between groups was conducted by Bonferroni post-hoc test. Data are represented in the form of bar graphs as (mean ± SD). Groups without shared letters indicate a significant difference between them (p-value < 0.05). All statistical analysis and graphs were performed using RStudiov1.3.1093 (RStudio Team, 2020) and R programming language v4.0.3 (R Core Team, 2020).

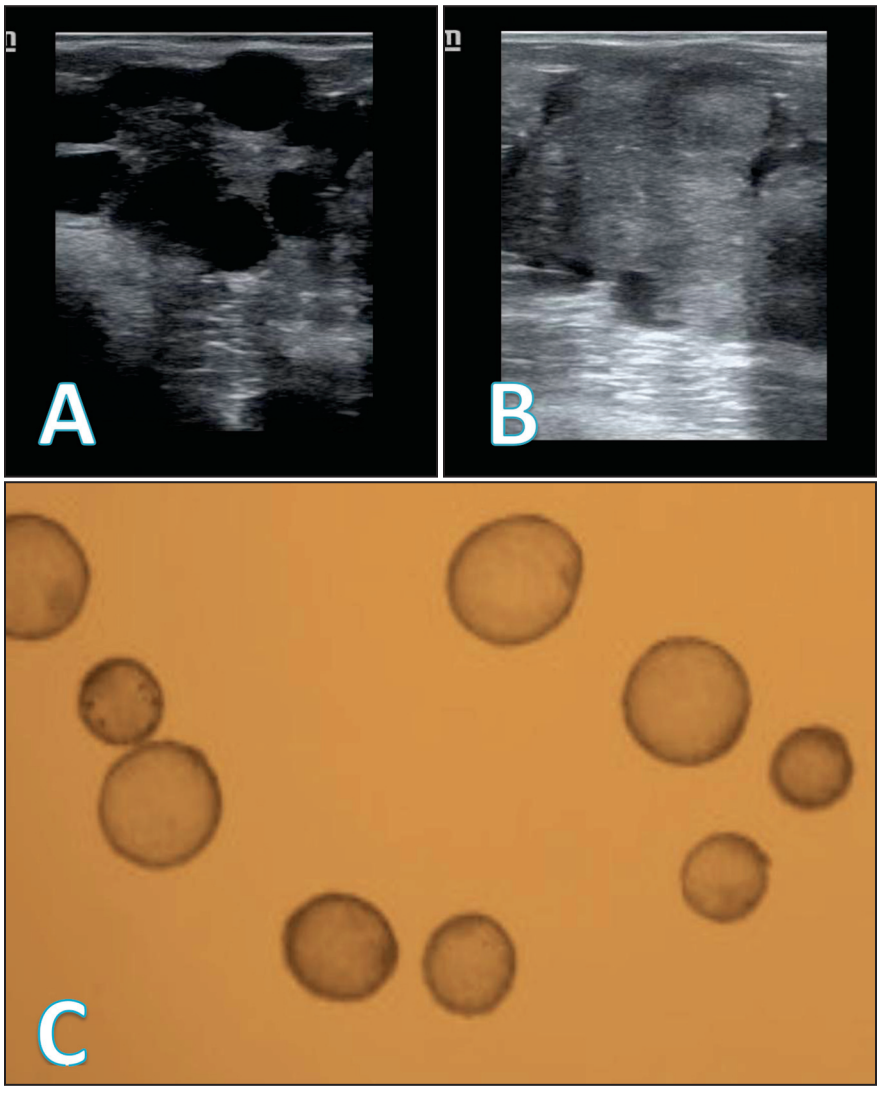

Fig 1. Representative pictures of A, multiple follicles as observed on the ovary after super-stimulation; B, Ovulated follicles on the same ovary as observed after breeding and C, Embryos flushed on D8 after breeding for transfer to synchronised recipients.

Results

The response of ovaries and embryo production in experiment 1 were summarised in Table 1. The proportion of mature follicles and the ovulations were similar (P> 0.05) in donors of first three treatment groups (Groups 2, 3 and 4), however, the donors in Group 5, which received a single s/c injection of 200 mg of FSH dissolved in aluminium hydroxide developed lower number of follicles and also had lower ovulated follicles when compared to other three groups and the control group (P< 0.05). The number of transferrable embryos was similar in group 2 and 4 (8.2±3.88; 8.3±2.44) and were not different (P > 0.05) from the control group (8.1±2.47), however, donors in group 3 and 5 produced lower number (P < 0.05) of transferrable embryos (4.9±2.47; 5.5±2.92) when compared to the other two treatment groups and control group.

We did not find any difference in the super-ovulatory response and embryo production among donors treated with FSH stored at room temperature (Group 2) or refrigeration (Group 3) when compared to control group as summarised in Table 2.

Discussion

To the best of our knowledge, this is the first study to investigate the effect of aluminium hydroxide, as a diluent for FSH, for its slow release after s/c administration, to induce ovarian super stimulation in dromedary camels. Regardless of the type of gonadotrophin product, its preparation or the protocols used for ovarian super-stimulation in camels, considerable variation have been reported in the super-ovulatory response in this species, which still remains one of the biggest challenges in application of embryo technology at farm level in dromedary camele (Anouassi and Tibary, 2013). Our results are encouraging and

Table 1. The effect of aluminium hydroxide, as a diluent to FSH, on ovarian super-stimulation and embryos production in dromedary camel (*Camelus dromedarius*) (Data expressed as mean ± SD).

Parameter	G1	G2	G3	G4	G5
Ovulatory follicles	21.1 ± 4.9[a]	22.7± 6.4[a]	19.1 ± 6.6[a]	17.7±3.7[a]	11.8±4.0[b]
Ovulations	19.5±4.6[a]	21.2±5.5[a]	17.6±5.7[a]	16±3.3[a]	10.7±3.2[b]
Un-ovulated follicles	1.6±0.9[a]	1.5±1.08[a]	1.5±1.1[a]	1.7±1.3[a]	1.1±1.1[a]
Total embryos	9.5±2.7[a]	9.5±4.1[a]	7.7±3.8[a]	10.1±2.7[a]	6.3±3.2[a]
Transferable embryos	8.1±2.4[a]	8.2±3.8[a]	4.9±2.4[b]	8.3±2.4[a]	5.5±2.9[ab]
Non- transferable embryos	1.3±0.8[ab]	1.3±1.1[ab]	2.8±1.4[a]	1.3±1.1[ab]	1.2±1.3[b]
Unfertilised ova	1.5±1.4[a]	2.1±3.6[a]	0.7±1.5[a]	0.3±0.6[a]	0.5±1.5[a]

Rows with different superscripts ae significantly different (P<0.05).

G1: Group 1, donors which received a traditional super stimulation protocol of 2500 IU of eCG and 400 mg of FSH is divided doses for 4 days

G2: Group 2, donors which received 2500 IU eCG as a single injection and 400 mg FSH (Folltropin V), dissolved in 10 ml of aluminium hydroxide (Imject alum) as a single subcutaneous injection.

G3: Group 3, donors received 2500 IU eCG as a single injection and 200 mg FSH (Folltropin V), dissolved in 10 ml of aluminium hydroxide (Imject alum) as a single subcutaneous injection.

G4: Group 4 donors received only as a single injection of 400 mg FSH (Folltropin V), dissolved in 10 ml of aluminium hydroxide (Imject alum) as a single subcutaneous injection.

G5: Group 5 donors received a single injection of 200 mg FSH (Folltropin V), dissolved in 10 ml of aluminium hydroxide (Imject alum) as a single subcutaneous injection.

Table 2. The effect of storing FSH, diluted in aluminium hydroxide, at room or refrigeration temperature on ovarian super-stimulation and embryos production in dromedary camel (*Camelus dromedarius*) (Data expressed as mean ± SD).

Parameter	G1	G2	G3
Ovulatory follicles	21.1 ± 4.9[a]	18.8± 3.4[a]	19.5± 3.9[a]
Ovulations	19.5±4.6[a]	17.2± 3.7[a]	17.5± 3.9[a]
Unovulated follicles	1.6±0.9[a]	1.2± 0.8[a]	2± 1.3[a]
Total embryos	9.5±2.7[a]	7.8±3.6[a]	7.5±2.9[a]
Transferable embryos	8.1 ± 2.4[a]	7 ± 3.9[a]	7.1 ± 3.1[a]
Non- transferable embryos	1.4 ± 0.9[a]	0.8 ± 1.0[b]	0.4 ± 1.0[b]
Unfertilised ova	1.5±1.4[a]	0.7± 1.5[b]	1.3± 1.2[a]

Rows with different superscripts ae significantly different (P<0.05).

G1: Group 1, donors which received a traditional super stimulation protocol of 2500 IU of eCG and 400 mg of FSH is divided doses for 4 days

G2: Group 2, donors received a reduced dose of FSH (200 mg) diluted in aluminium hydroxide and stored at room temperature for 14 days as a single subcutaneous injections.

G3: Group 3, donors received a reduced dose of FSH 200 mg diluted in aluminium hydroxide stored at refrigeration temperature for 14 days as a single subcutaneous injections.

demonstrate that a single subcutaneous injection of FSH diluted in slow-release diluent aluminium hydroxide gives similar results to that of our traditional protocol (control group) where FSH is injected two times per day in a decreasing dose for five days (P > 0.05). Using FSH diluted in saline in a traditional protocol injected twices per day for five days sometimes leads to muscle induration at the site of injection and increases the possibility of errors especially when the treated animals show irritable temperament. Also, it increases the stress on the animals because of handling that sometimes decreases the ovulatory response (Skidmore and Billah, 2005). The cost of hormones and manpower needed to administer these hormones in donors for super stimulatory treatments are major obstacles in widespread use of embryo transfer technology in camels. Some studies have reported that using a combination of FSH and eCG may lead to development of more than one generation of follicles, pre-mature luteinisation of follicle, decreased ovarian response or ovulation failure and increased cost of treatment (Anouassi and Tibary, 2013). The results of the present study showed that a dose of FSH reduced to half of traditional dose administered as a single s/c injection avoids all the above-mentioned side effects without jeopardising the number of follicles and embryos obtained per flush. Results of the present

study are similar to those reported by Manjunatha *et al* (2019), who used slow release preparation of FSH in divided doses for super stimulation in camels and found no difference in the ovarian response and ovulations when compared with traditional protocol.

The findings of this study are in agreement with earlier studies where it was found that using a combination of folltropin and equine chorionic gonadotropin increases ovulatory response and embryo production (Cooper *et al,* 1992; Nowshari and Ali, 2005). However, results are not consistent with these Ararooti *et al* (2018) who mentioned that FSH alone gives better results than combination of eCG and FSH.

In the current study, there were no significant ($P > 0.05$) difference in the super ovulatory response and embryo production among groups of donors receiving FSH diluted in aluminium hydroxide and stored at room temperature or 2-8°C for 14 days when compared to control group, who received FSH in traditional protocol. Further studies with a large sample size are planned to validate our findings.

Conclusion

This study investigated the effect of aluminium hydroxide, as a diluent for FSH, for its slow release after its administration subcutaneously, for inducing ovarian super stimulation in dromedary camels. We have developed a simple and cost effective protocol for ovarian super-stimulation in camels. The protocol uses a single subcutaneous injection of 200 mg of FSH diluted in aluminium hydroxide which produces similar results of the traditional protocol (400 mg of FSH in divided doses for 4 days, two times a day) for super ovulation in dromedary camels. The approach is easy to implement, cost and labour-effective, user-friendly and importantly, reduces stress on donor animals.

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