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Synchronization and superovulation of mature cycling gilts for the collection of pronuclear stage embryos

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Abstract

An efficient protocol was developed to synchronize and superovulate mature pigs for the collection of pronuclear stage embryos suitable for DNA microinjection. A timed and coordinated regimen of Lutalyse[®], PG600[®] and Chorulon[®] along with daily checking for estrus allowed synchronization of groups of gilts having estrous cycles at regular intervals. Pigs 10 to 16 days after the beginning of standing estrus have been successfully synchronized into estrus using this protocol. A standard dose of each drug was used independent of size or age of the animal. One protocol averaged 38.9 ovulations and 31.1 1-cell embryos recovered per animal.

Keywords

Gilt; Superovulation; Estrous Synchronization; Pronuclear stage embryo; PG600; Prostaglandin F_{2α}; Transgenic

1. Introduction

Timing of the reproductive cycle of embryo donors and recipients as well as superovulation are key components for the efficient production of transgenic pigs using DNA microinjection. Superovulation protocols used by us, and others (Pinkert et al., 1989; Williams et al., 1992; Petters et al., 1997; Maga et al., 2003) involved using prepuberal gilts. A continuous supply of prepuberal gilts was not available so groups of mature gilts (having estrous cycle at regular intervals) that ranged in age from 6 to 12 months were used in this study. Protocols using Regumate[®] (Wall et al., 1985; Pursel et al., 1997) were not considered because of space and housing limitations as well as personnel safety issues. We felt that by administering all drugs by injection would provide a safer working environment for the staff of our pig facility by ensuring containment and control over the reproductive medications.

Commercially available veterinary exogenous hormones were used to attenuate or supersede the normal estrous cycle and gain a high degree of control over the actual timing of ovulation as well as induce superovulation. PG600[®] is a drug composed of eCG (equine chorionic gonadotropin) and hCG (human chorionic gonadotropin) mixed at a ratio of 400 IU eCG: 200 IU hCG per 5ml. Chorulon[®] is a commercial form of hCG which can be used to mimic the LH surge in pigs and is used to induce ovulation. We also utilized Lutalyse[®], which is prostaglandin F_{2α}, to initiate a rapid luteolytic response. A variety of strategies were tried to synchronize and

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superovulate gilts. Adjustments of drugs utilized, as well as the doses and timing of their administration, were made to the protocols based on the ovulation response of the pigs.

2. Materials and methods

2.1. Gilts and detection of estrus

Six to 12 month old cross-bred gilts having estrous cycles at regular intervals were housed in groups of five or fewer. Animals were checked for estrus daily with an experienced boar and standing estrus recorded. The first day of standing estrus was regarded as day zero (Day 0) of the reproductive cycle. Protocols 1, 2, 3, 4 and 6 were carried out with gilts between Days 10 and 19 of the estrous cycle (Table 1). Protocols 5 and 7 used gilts with either undetectable estrus or gilts that were prepuberal. The animals with undetectable estrus were over 7 months old and either did not show, or quit showing lordosis and other signs during checking for estrus over several 3 wk intervals. The prepuberal gilts used were 163 to 170 days of age at the initiation of the protocol. All animal protocols were conducted in accordance with state and federal guidelines and were approved by the Institutional Animal Care and Use Committee (#04-057).

2.2. Synchronization and superovulation protocols

Protocols were investigated as independent observational groups and were not concurrent. All protocols were designed to begin recovery of 1-cell embryos at 1:00 pm on Wednesday (Table 1). The day of the pig's estrous cycle was determined by calculating the interval of days between the Friday administration of PG600 and the previous first day of estrus (Day 0). For example, a gilt listed as a Day 15 animal would have had its first day of standing estrus of its most recent cycle 15 days before the Friday administration of PG600. Artificial insemination (AI) was performed using fresh, pooled extended ejaculate from proven boars. Six billion sperm were delivered per insemination in 60 ml of BTS extender for each insemination. Inseminations performed between 7:00 AM to 11:00 AM were referred to as morning (am) breeding and inseminations performed between 1:00 to 3:30 PM were referred to as afternoon (pm) breeding.

2.3. Hormones used

Lutalyse® (Pharmacia & Upjohn Company, Kalamazoo, MI, USA) contains 5 mg/ml prostaglandin $F_{2\alpha}$ as the tromethamine salt. PG600® (Intervet INC. Millsboro, DE, USA) contains 400 IU eCG: 200 IU hCG per 5ml. Chorulon® (Intervet INC. Millsboro, DE, USA) contains 1000 IU hCG per ml. All injections were delivered intramuscularly. Doses given are found in Table 1.

2.4. Embryo recovery

Embryos were surgically recovered from the oviduct by retrograde flush with NCSU 23 medium (Petters and Wells, 1993). The number of corpora hemorrhagica (CH) were counted on the ovary. The flushed material was then observed using a Bausch and Lomb StereoZoom®7 stereomicroscope to count the ovulated oocytes and embryos.

2.5. Statistical analysis

CH, oocytes and embryos recovered and 1-cell embryos were analyzed by the general linear model (GLM) procedure of SAS (Cary, NC, USA) to determine differences among protocols and among day of the estrous cycle when the treatment was initiated. When a significant model effect was found for an experimental variable, data were compared by the LSD method. Statistical significance was determined as $P < 0.05$.

3. Results

Protocol 4 averaged the greatest number of ovulations (indicated by CH) as well as recovered oocytes and embryos (Figure 1A, 2A and Table 2) of all the protocols tested, but was not statistically different from protocols 2 and 3. Common between protocols 2, 3 and 4 was two injections of Lutalyse, the first injection being administered 16 hours before the second. Use of protocol 4 resulted in the greatest average for number of 1-cell embryos recovered of all the protocols tested (Figure 3A and Table 2); however, this was not statistically different from that for protocols 2, 3 and 6. Again, Lutalyse administration was common to these protocols with protocols 2, 3 and 4 receiving two injections of Lutalyse and protocol 6 receiving one injection. With protocol 1, gilts were exhibiting normal estrous cycles and did not receive Lutalyse and response was statistically different from protocols 4 and 2 in both ovulation rate and 1-cell recovery ($p<0.05$). These results suggest that Lutalyse administration is beneficial when used within the parameters of the present experiment.

Superovulation was defined for this study as 20 or more ovulations, which was determined by the number of CH present at the time of collection. Protocols 4, 2 and 3 each received two Lutalyse injections and respectively averaged 38.9, 32.6 and 28.6 CH and 31.1, 28.0 and 25.9 1-cell embryos per treatment (Table 2). The protocols that demonstrated superovulation were administered to animals with day of estrous cycle ranging from 10 to 16 d.

Protocol 1 consisted of use of gilts on 13 to 19 d of their estrous cycle that did not receive Lutalyse. These animals averaged 15.7 CH and 9.5 1-cell embryos (Table 2). Protocol 6 consisted of use of gilts on 12 to 13 d of their estrous cycle with gilts receiving one Lutalyse injection at the time of the PG600 injection. These animals averaged 18.4 CH and 15.4 1-cells (Table 2). With these protocols, estrous synchronization was achieved but not superovulation.

To determine which day of the reproductive cycle was best to initiate treatment, individual start dates across all treatments were compared (Figures 1B, 2B, 3B). For standardization, the start date is regarded as the day of the estrous cycle the pigs were in at the time of PG600 treatment. Over 20 CH per treatment day for 14, 15, 11, 13, 12 and 16 d (descending order) of the estrous cycle were observed (Fig. 1B). There was a 4-day window of the reproductive cycle where 20 or more 1-cell embryos per animal were obtained: 14, 15, 13 and 12 d (descending order) of the estrous cycle (Fig. 3B). It may be noted that the 11 d protocol resulted in a greater ovulation as observed for CH, oocytes and embryos recovered, however, the number of 1-cell embryos recovered was comparatively few (Figures 1B, 2B, 3B).

With protocol 7 prepuberal pigs, and protocol 5 mature gilts were utilized, respectively, that had no observable dates of estrus. When synchronization of estrus and superovulation was attempted in prepuberal gilts using a standard protocol of PG600 and hCG, synchronization of estrus was achieved but an average of 20 or more ovulations per animal was not achieved (Table 2). With protocol 5 mature gilts were utilized that for unknown reasons did not have a consistent or observable estrous cycle. When the treatment regimen of Protocol 3 was applied to these pigs, five animals total, 12.4 CH and 11.2 1-cell embryos were obtained (Table 2).

4. Discussion

The results indicate that it is possible to synchronize time of onset of estrus and superovulate gilts that were having normal estrous cycles for use in a oocyte microinjection based transgenic program. Daily checking for estrus is necessary. Acceptable results can be obtained without a constant input of prepuberal gilts or the need to sequester the pigs and feed Regumate. Common to the use of prepuberal animals or Regumate treatment in timed superovulation protocols is the coordination of the follicular phase of the ovary as well as controlling the dose and timing of follicular stimulation and ovulation with the aid of timed treatments. With prepuberal gilts

(over 100 d of age) the ovarian follicles are sensitive to hormonal stimulation but have not been induced to ovulate by endogenous endocrine action. Therefore, synchronization of ovulation with respect to exogenous gonadotropin delivery is permissible at any beginning time point. Regumate is used to maintain the luteal phase of the estrous cycle. After the Regumate is removed, the animals will resume having estrous cycles. eCG and hCG are used in coordination to synchronize ovarian folliculogenesis as well as synchronize the time of ovulation. The protocol in the present study, likewise, utilizes eCG and hCG in the basic strategy for synchronization of estrus and superovulation. The main difference is that the stage of the ovarian follicular phase was standardized in pigs having naturally occurring estrous cycles by administration of prostaglandin $F_{2\alpha}$ at a time in the reproductive cycle in which the CL is susceptible to luteolysis (Gleeson 1974, Hallford et al., 1975; and Guthrie 1976a).

Maternal recognition of pregnancy occurs from 10 to 12 d after estrus in pigs (Dhindsa and Dziuk, 1968). If there is no signal of pregnancy from embryos then the luteolytic phase begins and is complete by 15 d after estrus. What the protocol in the present study relies upon is the synchronization of luteolysis at a time when the CL is responsive to prostaglandin $F_{2\alpha}$ (Gleeson, 1974; Hallford et al., 1975; Guthrie and Polge, 1976a). Prostaglandin $F_{2\alpha}$ has been used to synchronize estrus in pigs having estrous cycles at normal intervals. To standardize the luteal phase in these pigs, accessory corpora lutea were induced at random stages of the estrous cycle by administration of PMSG followed with hCG 3 to 4 days later. Prostaglandin $F_{2\alpha}$ was then administered to synchronize estrus at least 12 days following the hCG treatment (Guthrie and Polge, 1976b; Guthrie, 1979). When eCG was given in combination with the prostaglandin $F_{2\alpha}$ treatment pigs had an improved response to estrus synchronization (Guthrie and Polge 1976b). Pigs having estrous cycles at normal intervals in which stage of estrus was synchronized with accessory CL induction and prostaglandin $F_{2\alpha}$ treatment were fertile and maintained pregnancy. The use of eCG followed by hCG allowed for timed insemination (Guthrie, 1979). We did not synchronize stage of the estrous cycle in pigs having estrous cycles at normal intervals by inducing accessory CL or by artificially maintaining the luteal phase of the cycle. We used Lutalyse to synchronize the ovarian stage (luteolytic phase) of pigs with naturally occurring estrous cycles ranging from 10 to 16 d of their cycle. It is also possible that the treatment of animals with Lutalyse at 15 or 16d is unnecessary, but, for convenience, all pigs were treated the same with regard to Lutalyse. Because the estrous cycle of pigs is about 19 to 21 days in length, with our protocol, we are able to synchronize estrus and superovulate one third of pigs with naturally occurring estrous cycles at any given time with the aid of data collected for detection of estrus.

We chose to use PG600 as the gonadotropin cocktail to stimulate ovarian follicular recruitment because it is commonly used on pig farms. PG600 is used on prepuberal gilts for synchronization and advancement of estrus (Guthrie 1977; Britt et al., 1989). PG600 is also used to advance estrus in weaned sows. When used at a constant dose, 400 IU eCG: 200 IU hCG, PG600 increases the rate of ovulation in pigs having naturally occurring estrous cycles in which stage of cycle was synchronized with Regumate as compared with prepuberal gilts that were pretreated with Regumate (Estienne et al., 2001). Gilts with naturally occurring estrous cycles at about 209 days of age (start of estrus in their herd was ~180d) treated with PG600 (5 ml dose 400 I.U. eCG: 200 I.U. hCG) had greater ($p<0.01$) ovulation rates than saline-treated control animals (28.8 vs 17.4 CL) when synchronization of estrus occurred with Regumate (Estienne et al., 2001). When prepuberal gilts (143 d of age) were pretreated with Regumate and then follicular development was induced with PG600, the ovulation rate was not statistically different from the sham-treated control group. Ovulation rate has been shown to be PG600 dosage dependent with prepuberal gilts and dose dependent based on the physical size of the prepuberal pig, as in the case of miniature pigs (Shimatsu et al., 2000; Breen et al., 2006). Because pigs having naturally occurring estrous cycles in the present study had variation

in size due to their variation in age, 6 to 12 months, a 1.5 x Dose of PG600 was selected to treat all animals.

Embryos from superovulated mature pigs have a greater developmental competence (Pinkert et al., 1989; French et al., 1991) than embryos from superovulated prepuberal gilts. Embryos obtained from pigs having naturally occurring estrous cycles are developmentally superior to those when superovulation treatments are used (Ziecik et al., 2005). However, due to problems inherent to collecting adequate numbers of stage-specific embryos for the procedure, it is not generally feasible to produce transgenic pigs via microinjection using naturally ovulated embryos. Because obtaining synchronized embryos in an efficient manner is important for producing pronuclear embryos for microinjection, methodologies that can facilitate this are necessary. We have previously produced transgenic pigs using superovulated prepuberal gilts as our sole source of embryo donors and recipients (Petters et al., 1997). We have now been successful in producing transgenic pigs from pronuclear microinjection of embryos in which stage of estrus was synchronized and superovulation induced with the protocols reported here using Lutalyse, PG600 and hCG administered to pigs having normally occurring estrous cycles (Estrada et al., 2006). Thus, the general utility of these protocols for synchronization of estrus and superovulation of mature pigs is evident.

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Fig. 1.

A & B. A - Protocol comparison of the presence of corpus hemorrhagica (CH) at the time of embryo recovery surgery. B - CH comparison by the start day of estrous cycle across all protocols. The day of the pig's estrous cycle is defined as the interval of days between the Friday administration of P.G.600® and the previous first day of standing estrus. PP = Prepuberal and NH = no detectable estrus. Different letter indicates statistically significant differences ($P < 0.05$)

Fig. 2.

A & B. A- Protocol comparison of recovered oocytes and embryos (recovered) averaged per animal. B - Comparison of recovered oocytes and embryos per pig by the start day of cycle across all protocols. The day of the pig's estrous cycle is defined as the interval of days between the Friday administration of P.G.600® and the previous first day of standing estrus. PP = prepuberal and NH = no detectable estrus. Different letter indicates statistically significant differences ($P < 0.05$)

Fig. 3.

A & B. A- Protocol comparison of 1-cell embryos averaged per animal. B - Comparison of 1-cell embryos per pig by the start day of estrous cycle across all protocols. The day of the pig's estrous cycle is defined as the interval of days between the Friday administration of P.G. 600® and the previous first day of standing estrus. PP = prepuberal and NH = no detectable estrus. Different letter indicates statistically significant differences ($P < 0.05$)

Table 1

Protocol schedule

	Thursday	Friday	Monday	Tuesday	Wednesday
Protocol 1 Mature 13–19d	--	10:00 am 5.0 or 7.5 ml PG600	11:00 am 750 or 1000IU hCG	2X AI am and pm	3:00 am ovulation* 1:00 pm embryo collection
Protocol 2 Mature 12–16d	3:00 pm 4.0 ml Lutalyse	10:00 am 7.5 ml PG600 4.0 ml Lutalyse	11:00 am 1000IU hCG	2X AI am and pm	3:00 am ovulation* 1:00 pm embryo collection
Protocol 3 Mature 11–16d	3:00 pm 4.0 ml Lutalyse	7:00 am 7.5 ml PG600 4.0 ml Lutalyse	7:00 am 1000IU hCG	2X AI am and pm* 11:00 pm ovulation	1:00 pm embryo collection
Protocol 4 Mature 10–15d	3:00 pm 4.0 ml Lutalyse	7:00 am 7.5 ml PG600 4.0 ml Lutalyse	7:00 am 1000IU hCG 1X AI pm	2X AI am and pm* 11:00 pm ovulation	1:00 pm embryo collection
Protocol 5 Mature no detectable estrus	3:00 pm 4.0 ml Lutalyse	7:00 am 7.5 ml PG600 4.0 ml Lutalyse	7:00 am 1000IU hCG	2X AI am and pm* 11:00 pm ovulation	1:00 pm embryo collection
Protocol 6 Mature 12–13d	--	7:00 am 7.5 ml PG600 4.0 ml Lutalyse	7:00 am 1000IU hCG	2X AI am and pm* 11:00 pm ovulation	1:00 pm embryo collection
Protocol 7 prepuberal	--	7:00 am 5.0 ml PG600	7:00 am 750IU hCG	2X AI am and pm* 11:00 pm ovulation	1:00 pm embryo collection

Protocol table. Columns of weekdays are in chronological order. Protocols were administered to groups of mature pigs 6–12 months of age, mature pigs with no detectable estrus, and prepuberal gilts. The day (d) of the pig's estrus cycle was determined by calculating the interval of days between the Friday administration of PG600 and the previous first day of heat (0d). Note that estrous synchronization was designed to recover pronuclear stage embryos beginning on Wednesday at 1:00 pm

AI = artificial insemination (am = 7–11:00am; pm = 1–3:30pm)

* Ovulation expected 42 h after hCG injection

Table 2

Protocol results

Protocol	CH Ave. (range)	CH=0	Rec Ave. (range)	Rec=0	1-cell Ave. (range)	1-cell=0	=20 CH
1 n=14	15.7 (0-33)	2	10.8 (0-28)	4	9.5 (0-28)	6	5
2 n=10	32.6 (6-52)	0	28.3 (0-65)*	1	28.0 (0-64) ₆	1	9
3 n=32	28.6 (7-52)	0	26.5 (0-67)*	3*	25.9 (0-67)*	3*	24
4 n=54	38.9 (9-73)	0	35.8 (3-83)	0	31.1 (0-73)	1	45
5 n=5	12.4 (0-22)	1	11.6 (0-29)	2	11.2 (0-29)	2	1
6 n=5	18.4 (6-34)	0	15.6 (4-34)	0	15.4 (3-34)	0	1
7 n=12	16.8 (6-29)	0	13.3 (5-28)	0	11.6 (5-20)	0	4

Various protocols were attempted to synchronize estrus and superovulate pigs. Results are recorded per treatment as the average of corpus hemorrhagicus observed, the average of oocytes and embryos recovered from the oviductal flush, and the average number of 1-cell embryos found at the time of collection. Superovulation is defined as at least 20 corpus hemorrhagicus present at time of collection.

CH = corpora hemorrhagica

Rec = recovered from flush: cumulus oocyte complex, 1-cell, and >1-cell

= 20 CH = considered superovulated

"= 0" is the number of animals in the protocol group that yielded a value of zero.

* n = 31 due to dropped collection dish