STUDIES ON SUPEROVULATION AND EMBRYO SEXING IN DAIRY CATTLE

By

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B.Sc., The University of British Columbia, 1987

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES
DEPARTMENT OF ANIMAL SCIENCE

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UNIVERSITY OF BRITISH COLUMBIA
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ABSTRACT

The presence of a dominant follicle has been shown to reduce response to superovulatory treatment. Studies were carried out to assess the superovulatory response to FSH/PGF$_{2\alpha}$ treatment using ultrasound scanning and progesterone profiles in the absence of a dominant follicle. The first experiment examined the effect of initiating superovulatory treatment at Day 2 of the estrous cycle, before a dominant follicle was identifiable. Although adequate follicular development occurred after administration of superovulatory hormones, few animals demonstrated estrus and ovulation rates and the number of embryos recovered was low. In the second experiment, human chorionic gonadotrophin (hCG) was used to remove the dominant follicle present at Day 7 of the estrous cycle prior to induction of superovulation on Day 9. Cows treated with hCG tended to have higher numbers of follicles, corpora lutea and embryos recovered after treatment, however values were not significantly different from control cows superovulated at mid-cycle.

Because the cost of superovulation and embryo transfer is high, economically it may be necessary to produce only calves of the desired sex. Several methods have been used to select for calves of the desired sex, including: production of sexed semen; determination of fetal sex in early pregnancy; and detection and selection of the sex of preimplantation bovine embryos prior to embryo transfer. Two methods of selection of
embryonic sex were investigated. Karyotyping was done to directly visualize the sex chromosomes of bovine preimplantation embryos. Previously, anti-H-Y antisera has been used in vitro and in vivo to select against male cells. Antibodies to H-Y antigen were produced in female mice after several weeks of immunization against male cells. Anti-H-Y antibody titre was assessed in enzyme-linked immunosorbent assays (ELISAs). Immunized females were then bred to study the effect of H-Y antibodies on litter size and sex ratio. Unexpectedly, increases in the male offspring were noted in the first litters of immunized females.
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ACKNOWLEDGEMENTS

I would like to thank the many people who helped with my projects over the last two and a half years:

Dr. Rajamahendran for being a supportive supervisor and source of good ideas, as well as helping with ultrasound scanning, mice bleeding and collection of embryos.

Chris Taylor and Collins Sianangama for performing most of the ultrasound scanning during the course of the projects.

The people in Dr. Lee's lab for help in maintaining cell cultures, developing ELISA systems and taking photomicrographs.

Sylvia Leung and Dr. Cheng for help with statistics.

Staff members at the UBC Dairy unit, who put up with my demands and interference.

My parents, who gave me encouragement.

B.C. Science Council which funded the H-Y antibody development project.
Chapter 1

Literature Review

A. Superovulation and Embryo Transfer

Superovulation is a method of treating a female with hormones to increase the number of follicles developing and thus increase the number of ovulations and eggs recoverable per female. Embryo collection involves surgical or non-surgical collection of ova from the female reproductive tract. Embryo transfer is the surgical or non-surgical procedure to place an embryo into the reproductive tract of a recipient. Superovulation in combination with embryo transfer can allow: a decrease in the generation interval, as females may be superovulated and embryos recovered before the animal could carry a pregnancy; progeny testing of females; use of superior females as embryo donors; increases in the number of progeny from superior females; the transport of embryos; research into early embryonic development; and study of maternal effects on embryo development (Foote, et al., 1970). The birth of the first embryo transfer calf was reported in 1951 (Willett et al., 1951). Since this time the embryo transfer industry has grown tremendously, such that 4% of the total calf registrations in the United States in 1987 were embryo transfer calves (Seidel, 1991). In addition, 27.5% of the top type-production index dairy cows in 1990 and 44% of the top indexing artificial insemination sires were born through the use of superovulation/embryo transfer techniques.
I. History of Superovulation

One of the first hormones that was discovered which can elicit superovulation was pregnant mare serum gonadotrophin (PMSG) by Cole and Hart in 1930. The hormone was discovered using a bioassay system in prepuberal rats. The rats were injected with varying quantities of serum from mares at different stages of pregnancy. When injected into prepuberal rats, PMSG was found to increase the numbers of follicles and ovarian weight and at higher doses could cause luteinization or ovulation of these follicles. The amount of serum required to produce these effects varied with the mare and stage of pregnancy. Since that time, PMSG has been widely used to elicit superovulation responses in cattle.

Willett et al. (1948) investigated the effect of timing of initiation of superovulation in relation to the day of the estrous cycle, the amount administered and the hormone used for ovulation induction in the superovulation of heifers. The regimen used to cause superovulation consisted of one injection of follicle stimulating hormone (FSH) subcutaneously daily for five days, followed by ovulation induction on the sixth day and artificial insemination was performed on the sixth and seventh day. No fertilized ova were recovered from heifers which began hormonal treatments on day 4 of the estrous cycle. Failure was attributed to uterine infections caused by attempting to perform artificial insemination in the luteal phase of the cycle. The best responses to superovulation induction occurred when treatment was initiated in the follicular phase of the estrous
cycle. Heifers treated on day 16 with FSH extracted from 30 or 40g of sheep pituitaries had better responses than those treated with 20g.

In a review, Foote et al. (1970) noted that many regimes of hormone treatment had been tried to induce superovulation, the most successful being the use of PMSG or porcine FSH. Better ovulation rates were observed if the superovulation treatment was conducted in the absence of an active corpus luteum (CL). The best method to avoid an active CL was to begin superovulation treatment on day 15-16 of the estrous cycle to or manually or surgically remove the CL. Avery et al. (1962a) used purified porcine FSH and luteinizing hormone (LH) to stimulate superovulation in calves, cows and pregnant animals and was able to induce ovulations in 61 of 75 animals treated. For cows, the dosage used was 20mg FSH and 5mg LH subcutaneously on the first day, 10mg FSH and 5mg LH on the second day, 10mg FSH and 5mg LH on the third and fourth days, and 100mg LH i.v. on the fifth day. Twenty-eight of thirty-two cows ovulated in response to treatment. The use of progestogens to synchronize the cycle or removal of the CL caused better estrus responses. Hafez et al. (1963) used 3000i.u. PMSG followed by 2000i.u. hCG 5-6 days later to superovulate cows. Estradiol-17β (E₂) given after PMSG increased the number of follicles ovulating. The protocol used for superovulation consisted of 3000i.u. PMSG on day 16 of the cycle, 20mg E₂ on day 19 and 20 and 2000i.u. human chorionic gonadotrophin (hCG) on day 21. Hafez et al. (1963) also found that performing several inseminations ensured better
fertilization rates. Scanlon et al. (1968) superovulated cows with 3000i.u. PMSG on day 16 followed by 2000 i.u. hCG at estrus or 5 days after PMSG if estrus was not seen. Of eighty-nine cows treated, 88 were successfully superovulated, however only 79/89 had shown estrus. When the reproductive tracts were flushed at slaughter, 74% of the cows treated produced more than one egg, and the eggs recovered represented 70% of the corpora lutea. From the 79 cows which showed estrus, an average of 4.4 fertile eggs were recovered. Foote et al. (1970) noted that for good responses to superovulation, FSH had to be given once or twice a day for five days while PMSG could be given in one injection.

Early work in attempting to increase the efficiency of beef production by hormonal induction of twinning increased knowledge about superovulation. Bellows et al. (1968) attempted to produce multiple births in progestogen- synchronized beef cattle using several regimes of hormone treatment. The treatments consisted of 75mg FSH in one injection; twice daily injections at constant doses for a total of 6.25-75mg FSH; or a decreasing dose schedule twice daily for a total of 50mg FSH. Laparotomies were performed to count the number of ovulations. Fifty mg FSH produced an average of 17.8 CL at a constant dose and 23 CL when administered at a declining dose. Injected twice daily, FSH produced a greater response than the same amount of FSH given in one injection. Total dosages of 25-75mg FSH produced similar effects, although there was a large amount of variability at each dose. Twenty-five mg FSH produced an average of 13.0 CL
with a range of 1-30 CL. A total dosage of 6.25mg FSH produced an average of 2.1 CL with good fertilization rates of eggs, and in this experiment was the best dosage for induction of twinning. Laster et al. (1970) induced twinning using 2000i.u. PMSG on day 5 and 1500i.u. on day 17 of the estrous cycle and found that 80% of the synchronized heifers treated had 1-3 CL but 13% had over 6 CL. Laster et al. (1973) compared PMSG and FSH in the induction of twinning in heifers. The number of CL was higher and more variable with FSH, ranging from 1-19, and the average was 4.4 ± 4.9 CL vs. 1.6 ± 1.0 CL after PMSG treatment. Many of the heifers aborted, which was probably due to carrying excessive fetuses.

Researchers have noted that there is a lot of variation in superovulation response to a given amount of hormone, and thus many attempts have been made to investigate the causes of variation in response. Elsden et al. (1974) suggested that the highly variable responses were likely related to the difficulty in selecting the time of administering PMSG in relation to the next predicted estrus. Rowson et al. (1972) were among the first to use prostaglandin in combination with superovulation treatment. Prostaglandin F$_2$ (0.5mg) could be administered on 2 consecutive days into the uterine horn adjacent to the corpus luteum on day 5-16 of the estrous cycle and would induce estrus usually by the morning of the third day. Rowson et al. (1972) found this to be an efficient method of synchronization of estrus and resulted in normal fertility.

Elsden et al. (1974) administered 1500-2000 i.u. PMSG in
the mid-luteal phase of the cycle, followed 48h later by 1mg doses of prostaglandin \( F_{2\alpha} \) (PGF\(_{2\alpha}\)) for two consecutive days into the uterine lumen ipsilateral to the CL. Eighteen of twenty-four cows exhibited heat within five days of PMSG treatment, and all 24 ovulated to produce an average of 13.2 ± 1.9 CL. Of 35 cows which were given 2000i.u. PMSG alone on day 16 of the cycle, only 24 had responded to treatment and only 17/24 had ovulated for an average of 8.0 ± 1.5 CL. Use of PGF\(_{2\alpha}\) to synchronize estrus in superovulation protocols, increased the number of cows which ovulated, increased the number of ovulations per cow and resulted in good fertility. Prostaglandin \( F_{2\alpha} \) is now an integral part of superovulation and estrus synchronization regimes in cattle.

II. History of Embryo Collection and Embryo Transfer Techniques

The first successful embryo transfer was reported in rabbits in 1890 (Heape, 1890). After that time few successes were reported until almost the 1950’s. In 1949, Warwick and Berry reported experiments done using embryo transfer in sheep and goats. Goat embryos surgically transferred to the sheep uterus did not survive past 22 days, although sheep embryos survived for at least 45 days in the goat uterus but none survived to term. Some sheep-sheep and goat-goat transfers did survive to produce live young. Both saline and Tyrode’s medium were used for transfers with limited successes, then the use of aqueous humour from the eye discovered to be a better transfer medium.
Chang (1950) reported the importance of synchrony between the stage of the embryo and stage of the recipient’s estrous cycle. One day old rabbit embryos could only be successfully transferred to oviducts. However, two day old rabbit embryos could be transferred either to a two day post-estrus uterus or two day oviducts, but not a one day uterus. Four day old embryos could only be successfully transferred to the uterus, but could tolerate up to two days of asynchrony (i.e. two to six day post-estrus uterus). Chang (1950) used homologous serum alone or a mixture of 1:1 serum and saline for embryo collections and transfers. Similar requirements for synchrony between the stage of embryo and recipient uterus was later noted in cattle. Newcomb et al. (1975) found that few pregnancies were achieved after transfer of two or three day old bovine embryos into the uterus, but four day embryos could tolerate at least ±1 day asynchrony when transferred into the uterus. The necessity for synchrony was believed to be an embryo requirement for a progesterone-primed uterus and for appropriate signalling between uterus and embryos to initiate pregnancy.

The first successful embryo transfer (E.T.) in cattle was reported in 1951 (Willett et al., 1951). The estrous cycles of donors and recipients were synchronized with progestogens. Embryos were recovered at slaughter from superovulated donors and were flushed from the reproductive tracts using homologous blood serum. Embryos were surgically transferred in serum to a recipient through mid-ventral laparotomy and placed into the uterine horn using a glass micropipette. Two more calves were
born using the same method in 1953 (Willett et al., 1953).

Most of the early efforts to collect embryos for embryo transfer were done by flushing the reproductive tract after slaughter of the donor cow, although some embryo recoveries were performed surgically. Several attempts were then made to design non-surgical methods of embryo collection to lower costs; to increase the repeatability of the procedure, because multiple surgeries caused adhesions of the reproductive tract; to reduce the time required to complete the collection, and to reduce the period of recovery for the animals. Dracy and Petersen (1950) used two methods to attempt to collect fertilized embryos. In the first method, a rubber catheter was passed through the cervix through the uterine horn to the utero-tubal junction, to attempt to catch the egg as it passed from the oviduct into the uterus. This method was unsuccessful because the uterus underwent violent contractions to force the catheter out and at least one egg was able to bypass the catheter and cause pregnancy. The second method, used to collect embryos from the uterus seven days after estrus, was more successful. A probe was used to dilate the cervix and then a steel cannula was passed into the uterus and directed to either uterine horn. A bicycle pump was used to flush one litre of warm physiological saline into the uterus. The returning fluid was collected and allowed to settle in separation funnels before eggs were located. In 37 flushes, embryos were recovered 12 times. Dziuk and Petersen (1954) attempted non-surgical embryo collection and embryo transfer. A self-retaining catheter with 10 holes was
placed into the uterus and 200ml of homologous serum was used for flushing. In 13 attempts to collect embryos, five collections were successful. Elsden et al. (1976) reported success using non-surgical embryo recovery. The cow was prepared by giving epidural anaesthesia, clearing the rectum of feces, tying the tail out of the way and washing the perineal area. A cervical expander was placed into the cervix then a Foley catheter with large holes, stiffened with a metal stylette, could be guided into the uterus. The balloon on the Foley catheter was inflated with 20-30ml air and the stylette removed. Eight hundred millilitres of phosphate buffered saline (PBS) with 1% serum was used for flushing the uterus, and the fluid recovered was collected into 250ml sedimentation funnels. Elsden et al. (1976) was able to collect an egg from 36/51 unsuperovulated donors. Twenty-four of twenty-six attempts to collect embryos from superovulated donors were successful. Although it is more difficult to locate eggs due to the large volume of flushing medium, the non-surgical technique is more efficient than surgical methods of embryo collection because it may be used repeatedly, causes little damage and can allow single egg collections between superovulations. The non-surgical method of embryo collection is now used almost exclusively, with only a few modifications from the original procedure.

There were also several attempts to design methods to achieve non-surgical embryo transfers with similar reasons as for performing non-surgical recoveries. Avery et al. (1962b)
tried several methods of embryo transfer. Non-surgical embryo transfers were done by three methods: a) the use of an artificial insemination pipette to pass through the cervix on day 4, and air displacement of the embryo in a small volume of fluid; b) the use of a steel cannula to pass through the cervix and then passage of a capillary tube through the steel cannula and displacement of the embryo in a small volume of fluid; c) the use of a long needle to puncture the rectal wall then the uterine lumen. Tubing and a small needle were passed through the long needle and the embryo displaced using a 20mL syringe. No pregnancies resulted through any of these procedures. However, one pregnancy was achieved after four surgical transfers by laparotomy. Sugie et al. (1965) designed one of the first successful methods for nonsurgical embryo transfer. Three plastic tubes were designed which fit inside each other. The A tube was placed into the vagina and was directed adjacent to the cervix. The B tube, which had a long hypodermic needle, was guided so that the needle punctured the vaginal wall and entered the uterine lumen. The C tube had a small diameter hypodermic needle which contained the embryos, the embryos were displaced by slight pressure on the bulb. Carbon dioxide gas was then passed into the uterus to stop uterine contractions. Two pregnancies and one live calf resulted from this method of non-surgical transfer. The use of a similar apparatus in five goats produced four pregnancies. The advantage of Sugie's method of non-surgical transfer was that it avoided passing through the cervix. Rowson et al. (1966) reported that the two main causes for failure to establish pregnancy after nonsurgical
embryo transfer were the high rates of inducing uterine infections during manipulations and by expulsion of the transferred eggs via the cervix due to violent contractions of the uterus. Inflation of the uterus with CO\textsubscript{2} allowed better retention of transferred eggs. Successful non-surgical transfers were achieved by passing a sterile speculum through the cervix, followed by deposition of 2-3 eggs in 0.5mL serum using an artificial insemination pipette. The uterus was then distended with CO\textsubscript{2} gas. In six transfers with more than two days asynchrony, no pregnancies resulted. However, when synchrony was ±1 day, 3 pregnancies from 8 transfers were achieved. Rowson et al. (1969) reported that the method of collection and transfer, medium used for transfer and synchrony were important in establishing pregnancies through embryo transfer. Of 33 eggs transferred in serum, half surgically and half non-surgically, no pregnancies resulted. When embryos were transferred in tissue culture medium (TCM) using surgical embryo transfer, more pregnancies resulted from embryos collected surgically than from embryos collected after slaughter of the donor. A pregnancy rate of 20% was achieved after non-surgical embryo transfer.

Most embryo transfer procedures are now done nonsurgicaaly. Non-surgical embryo transfers are currently performed using an embryo transfer pipette which is much the same as an artificial insemination pipette, except that it is longer so that embryos may be placed deeper into the uterus. Similar procedures are used to prepare recipients as for donors at embryo collection. An epidural block is now used to anaesthetize the uterus instead
of inflation with CO₂ gas. The tail is tied out of the way and the vulva washed. Then the E.T. pipette is passed through the cervix into the uterine horn ipsilateral to the corpus luteum and the plunger depressed to release the embryos. Over the years, pregnancy rates from non-surgical transfer have improved to 50-60% (Picard et al., 1985).

III. Variability of response to superovulation induction

Superovulation and embryo transfer are used widely for research and as a method to increase the number of progeny from superior females. Despite advances in the last twenty years in developing better drugs for superovulation induction, techniques for non-surgical embryo collection and transfer; superovulation is still hampered by the unpredictability of superovulation response. After discovery and widespread use of prostaglandin F₂α or its analogues to control timing of estrus in superovulated cattle; the biggest problem which remained in inducing superovulation was the high variability in response to treatment. Donaldson (1984) reported in over 1200 superovulation attempts that one third of the donors treated produced no transferrable embryos.

Response to superovulation depends on many factors, both extrinsic and intrinsic to the animal. Response to superovulation is known to depend on the amount of drug administered (Bellows et al., 1969); the type of drug used, response to FSH is generally higher than PMSG (Monniaux et al., 1983); the purity of the drug preparation, as high amounts of LH
contamination in FSH preparations decrease superovulation response (Murphy et al., 1984); schedule of administering the drug, giving FSH 2 or 3x daily produces better responses than once daily injections (Chupin and Procureur, 1982); and giving FSH at a decreasing dose produces better responses than a constant dose (Bellows et al., 1969; Chupin and Procureur, 1982).

Intrinsic factors also influence superovulation response. Romero et al. (1991) found that the number of small follicles on the ovaries before treatment affected the number of follicles generated after hormonal induction of superovulation. Monniaux et al. (1983) noted that one factor which affects superovulation success is the ovarian status at the time of initiation of treatment. Several researchers have attempted superovulation induction at different times of the cycle. Philippo and Rowson (1975) administered PMSG, in combination with PGF$_{2\alpha}$, at four different times of the cycle and found the best responses when superovulation was initiated between Days 8-12. Sreenan and Gosling (1977) and Lindsell et al. (1985) and also found better results when superovulation was initiated on Days 8-12 of the estrous cycle.

Much research has been conducted into studying follicular growth and atresia in cattle. Moor et al. (1984) reported that most of the small follicles present in the ovary are atretic. Monniaux et al. (1983) reported that superovulation does not increase the number of small follicles present in the ovary, but
instead rescues follicles normally destined for atresia. Rajakoski (1960) studied follicular populations in heifer ovaries recovered at slaughter from animals at known stages of the estrous cycle and concluded that follicular growth occurs in waves. However, early work on follicular dynamics was hampered by lack of a method to repeatedly survey follicular development in individual animals and thus relied on specimens recovered after slaughter or after laparotomy. Matton et al. (1981) performed laparotomies to mark the largest follicles present on the ovaries with india ink. At slaughter a few days later, the fate of the marked follicles was determined. It was concluded that there were several periods of follicle turnover during the estrous cycle and that early in the estrous cycle there was little or no turnover of the largest follicle. Ireland and Roche (1983) examined ovaries collected at slaughter at known days of the cycle and classified follicles > 6mm histologically and endocrinologically as healthy or atretic. It was found that there were several periods of follicular growth. On days 3-7 of the estrous cycle, all heifers had one large non-atretic follicle in a pair of ovaries, but by day 9-11 only one large atretic follicle was found. After day 13 another large non-atretic follicle was found. Moor et al. (1984) reported that medium sized follicles are most common days 0-5 and 9-13 of the cycle, and this may explain why superovulation response is best on days 8 to 10 of the estrous cycle.

Recently, ultrasonography has allowed monitoring cattle ovaries on a frequent, often daily, basis. Pierson and Ginther
(1984) first reported monitoring follicular growth throughout the estrous cycle, and concluded that the numbers of follicles in different size classes varied with day of the estrous cycle. Pierson and Ginther (1987) reported that there were two waves of follicular activity in heifers. Savio et al. (1988) and Sirois and Fortune (1988) reported that most heifers have three waves of follicular growth; however most cows have two waves (Taylor and Rajamahendran, 1991). A follicular wave is characterized by the appearance of a cohort of small follicles, followed by the selection of one follicle which becomes dominant and continues to grow to preovulatory size while the rest of the cohort regress. The first dominant follicle can be detected on day 4, reaches maximum size on day 6 and remains stable between days 6-10. Then the first dominant follicle begins to regress and is not detectable by day 15. A second dominant follicle is detectable by day 12, reaches maximum size on day 16, and will ovulate in a two wave cycle. In three wave cycles, the third dominant follicle is detectable on day 16, grows to maximum diameter on day 21 and ovulates (Savio et al., 1988).

Ultrasonography has been used to monitor follicular development and corpus luteum formation during superovulation induction (Pierson and Ginther, 1984; Goulding et al., 1990). Goulding et al. (1990) have postulated that the current optimal time to initiate superovulation treatment is at mid-cycle, when an active dominant follicle may not be present. However the exact timing this period may vary depending on whether the cycle would have two or three waves of follicular growth. Recently,
the presence of a dominant follicle at the time of induction of superovulation has been shown to decrease superovulatory responses (Guilbault et al., 1991; Huhtinen et al., 1992). Grasso et al. (1989) stated that the presence of a dominant follicle at the initiation of hormone treatment will both decrease the number of large follicles stimulated by the treatment and delay the appearance of these large follicles. However, Wilson et al. (1990) did not find any effect of a dominant follicle on superovulatory response.

B. Methods used for semen and embryo sexing to produce offspring of the desired sex

Normally the sex ratio at birth is approximately 1 male:1 female. However, the ability to choose the sex of offspring has been desired for many years both in the human population, and for important livestock species. In livestock, females may be desired for herd replacements, while in other situations, males may be preferred for more efficient meat production or for sale contracts with artificial insemination organizations. In mammals, males are the heterogametic sex. When an X-chromosome bearing spermatozoa fertilizes the egg, a female offspring will result, but when a Y-chromosome bearing sperm fertilizes, a male will result. Therefore, the most efficient place to act in order to produce embryos and pregnancies of the desired sex is to selectively increase the numbers or impair the function of either the X- or Y-chromosome bearing sperm. In practical and economic terms, semen would have to be enriched to produce >80% males or females (Pinkel et al., 1985). Many attempts have been
made to alter the sex ratio by selecting for the sex of sperm prior to fertilization.

I. Methods to separate X- and Y-spermatozoa

Modifying pH of the female reproductive tract

One of the earliest used methods to select for the sex of the fertilizing spermatozoa came from attempts to alter the pH of the female reproductive tract by using acidic or basic douches. Increases in the numbers of females born have been reported after acidic douches and increased males after basic douches in rabbits (Roberts et al., 1940). Using similar pH protocols, no sex ratio differences have also been reported in rats or rabbits (Cole et al., 1940).

Density gradients

The X-chromosome is one of the largest of the chromosomes but the Y-chromosome is among the smallest chromosomes in mammals. Therefore sperm which carry the X-chromosome, may be heavier than sperm which carry a Y-chromosome. Sumner et al. (1976) found that sperm had two peaks of dry mass. Because Y-sperm carry less DNA, it is believed that they may be more motile (Goodall et al., 1976) or less dense, therefore there have been many attempts to separate X- and Y-sperm based on these two characteristics. The most commonly employed method has been the use of albumin gradients. The use of a discontinuous gradient or several layers of bovine or human serum albumin are believed to select for Y-sperm, as these sperm should be able to move faster and farther into the viscous
medium. Many researchers have claimed success using this method. A commonly used method in which 2- or 3-layers of albumin are employed to separate spermatozoa, has been used in several clinics in the United States to select for Y sperm (Ericsson et al., 1973; Dmowski et al., 1979; Beernink et al., 1982) to increase male births. Other researchers demonstrate no separation in X- or Y-chromosome bearing sperm (Evans et al., 1975; Ross et al., 1975). The use of bovine serum gradients to select for sex in cattle has been attempted but with little success (Beal et al., 1984). Numerous other types of density gradients have been used to try to separate X- and Y-sperm through expected differences in swimming behavior or by centrifugation in Percoll (Iwasaki et al., 1988; Upreti et al., 1988), egg yolk (Schilling et al., 1966) or Sephadex (Quinlivan et al., 1982) but with no consistent effects on sex ratio. An attempt was made to filter sperm through cervical mucus and claimed to increase the proportion of male sperm but not enough to enrich X- or Y-sperm populations (Broer et al., 1978).

**Surface charge**

Several attempts were made in attempting to separate X- and Y-sperm via electrophoresis or galvanization, on the basis of possible differences in surface charge. Some workers have found increased numbers of Y-sperm at the anode (Shishito et al., 1975; Bhattacharya et al., 1977) while others did not (Hagele et al., 1984). Many other experiments were designed to separate sperm through thermal convection or laminar flow through supposed differences in swimming behavior (Hagele et al., 1984;
Sarkar et al., 1984), also with no consistent success.

**H-Y antigen**

In 1955, a male-specific antigen coded by the Y-chromosome was discovered to be present on male mouse skin (Eichwald and Silmser, 1955). Since that time the antigen, H-Y antigen, has been localized to many mammalian male tissues including spermatozoa (Goldberg et al., 1971). It has been believed that because male determining sperm have the Y-chromosome which encodes the H-Y antigen gene, that more H-Y antigen may be present on Y-sperm. There have been several attempts to alter the sex ratio by combining antisera raised against H-Y antigen with sperm prior to insemination with some reports of increases in the number of females born (Bennett and Boyse, 1973; Zavos, 1983). However others, (Hoppe and Koo, 1984) found no differences in sex ratio when sperm had been reacted with monoclonal anti-H-Y antibodies and complement prior to use in in-vitro fertilization of mouse eggs. Some experiments have tested the effect of maternal H-Y immunization in vivo. Immunized females have been bred and produced increases in females (Pechan, 1985; Singh and Verma, 1988), no change in sex ratio (McLaren, 1962; Rao et al., 1981) or increases in males if the immunized female had been previously splenectomized (Lappé and Schalk, 1971; Shalev et al., 1980). It therefore seems unlikely that H-Y antigen is expressed on Y-sperm preferentially. In fact, H-Y antigen expression on sperm has been found to decrease as the sperm ages (Hoppe and Koo, 1984), therefore maturational state of sperm could explain differences
in H-Y antigen expression.

**Evaluation of X- and Y-chromosome bearing sperm separation**

Some of the difficulties in measuring whether there have been significant shifts in the proportions of X- and Y-sperm, lie with the methods used to measure separation. Differences are known to exist between X- and Y-spermatozoa in mammals. Pearson et al. (1970) discovered that the human Y chromosome stains very brightly with the fluorescent dye quinacrine mustard. This bright spot is labelled the fluorescent or F-body. Approximately 50% sperm carry the F-body (Barlow and Vosa, 1970) and thus the presence of an F-body has been widely used to quantify the number of sperm with Y-chromosomes in human sperm sexing regimens. However, researchers report that often less than 50% sperm exhibit F-bodies, and also F-bodies are not found in all species (Hagele et al., 1984; Ogawa et al., 1988). Counting the number of sperm with F-bodies may be rather subjective. Also other chromosomes such as human chromosome 3 and the D group chromosomes (Pearson 1970) also show areas of intense fluorescent staining which may be confused with the F-body.

**Zona-free Hamster Oocyte assay**

Rudak et al. (1978) developed the zona-free hamster oocyte assay to be able to visualize sperm chromosomes. Hamster oocytes, after removal of the zona pellucida, can be penetrated by sperm of other species. After several hours of culture, the sperm head will decondense and the chromosomal complement of the
sperm can be analyzed. Tateno et al. (1987) used zona-free hamster oocytes to study the proportions of X- and Y- bull sperm after attempts at sperm separation. They found that 73-92% hamster oocytes were penetrated, with about 42% of the eggs having analyzable sperm chromosome complements. Due to the high incidence in polyspermy, for 100 eggs there were 56 analyzable sperm complements.

**Flow Cytometry**

Mammalian sperm differ in the amount of DNA they contain, because there is a large difference in X- and Y-chromosomal size. Therefore it may be possible to separate sperm on the basis of the amount of DNA. Some fluorescent dyes are known to bind stoichiometrically to DNA. The use of a fluorescence-activated flow cytometer to measure the amount of DNA in sperm involved several modifications. The flow cytometer required modifications in order to control the introduction of sperm so that orthogonal flow was achieved, to reduce the refraction due to sperm head shape and orientation (Van Dilla et al., 1977). Unfortunately, to achieve good separation of peaks with high (more DNA, X-sperm) and lower fluorescence (less DNA, Y sperm), which only differ by 3-4% in total DNA, several modifications of the sperm were necessary. Sperm have been treated with DMSO, ethanol, papain and dithioerythritol to allow stoichiometric fluorescent DNA stain uptake as well as removing sperm tails to allow better orientation (Otto et al., 1979; Garner et al., 1984). These treatments are quite harsh, and leave sperm largely nonviable. These sorted spermatozoa may be used if
microinjected into oocytes, although only a low proportion of eggs will become activated (Johnson et al., 1988a). More recently, methods have been modified in using sonication and a stain which is less damaging to the sperm (Johnson et al., 1987). Sperm which were sorted once and then resorted (due to difficulty in getting good orientations with intact sperm with tails) produced X-sperm with 86% purity and 81% purity of Y-sperm (Johnson et al., 1989). These sperm were used for intrauterine insemination of rabbits and produced 94% females from the X-sorted sperm and 81% males from the Y-sorted spermatozoa. However, Johnson et al. (1989) state that the cost of the flow cytometer is too high and the semen sorting speed too slow to produce large amounts of sexed semen for artificial insemination. Morrell et al. (1988) report pregnancy rates to be low using flow-sorted semen. However, flow cytometry may be used successfully to quickly produce counts on the percentages of X- and Y-sperm in semen samples that have been separated using other sexing methods, thus could save the time and expense in performing fertility trials. Flow cytometry has been used to count numbers of X- and Y-sperm from sperm separated by albumin, laminar flow, Percoll, Sephadex, H-Y antibodies, magnetism and electrophoresis (Pinkel et al., 1985; Johnson et al., 1988b). In over 200 samples analyzed, no differences in X:Y ratio have been reported. Although flow cytometry can not measure any differences in fertilizing capacity of X- and Y-spermatozoa in sexed semen; no method of semen sexing tested has reliably changed the relative proportions of X- and Y-sperm.
II. Methods to detect the sex of the fetus in early pregnancy

Some methods have been used successfully to determine the sex of the fetus during early pregnancy. However, determining the sex at this time may be quite an inefficient process because by this time the pregnancy is well underway and time and money will be lost in inducing abortion of fetuses of the wrong sex and trying to get the female pregnant again.

Hormonal Determination

One of the methods used to determine the sex of the fetus, is by obtaining allantoic fluids to measure the concentration of testosterone (Bongso et al., 1976). Testosterone levels in allantoic fluid of over 320pg/mL indicate the presence of a male fetus in cattle.

Karyotyping

Another method to sex the fetus, is by collecting amniotic fluid. Cells contained in the amniotic fluid must be cultured until there are enough to perform chromosomal analysis. If fluid is collected at d70-90 of pregnancy, results could take 2-3 weeks (Hare et al., 1978).

Use of Ultrasound

Ultrasonic evaluation of fetal sex, by attempting to visualize scrotal swellings of the male, can be done in cattle after day 57 of pregnancy. Muller et al. (1986) reported this method to be very accurate.
**Use of DNA probes for the Y-chromosome**

Chorionic biopsy is a technique that has been applied to humans and may be useful in sexing cattle pregnancies. DNA probes have developed which are specific for the Y-chromosome of man (Kunkel et al., 1976; Bishop et al., 1983; Handyside et al., 1989). Others have been developed for identification of the Y-chromosome in mice (Nishioka et al., 1986) and cattle (Leonard et al., 1987). Gosden et al. (1984) used the techniques of chorionic biopsy and in situ Y-probe hybridization to sex human fetuses and had the results within a few days.

**III. Methods of sexing preimplantation embryos prior to embryo transfer**

Due to the facts that semen sexing is unreliable and sexing the fetus during pregnancy is inefficient; recently more effort in obtaining pregnancies of the desired sex has focused on detecting the sex of preimplantion embryos prior to embryo transfer. In 1987, 4% of the total U.S. cattle registrations were born through embryo transfer techniques (Seidel, 1991). Therefore, there may be a relatively large market for sexing pre-implantation embryos. A catalogue from British Columbia Artificial Insemination Centre (1990) advertises frozen embryos for sale at three hundred dollars each. The cost of embryos is high, therefore it may be well worth the money to ensure that the desired sex of calf will be born. Some commercial embryo transfer companies offer embryo sexing (Bondioli et al., 1989) as a service. There are two main categories of sexing embryos; noninvasive and invasive techniques.
Non-invasive techniques

Evaluation of cleavage rates

Non-invasive methods of embryo sexing are less detrimental to the embryo, usually easier to perform, but may be less accurate. One method to assess sex of embryos non-invasively is to examine the stages of embryos recovered at collection. Due to the fact that males are heavier than females at birth and thereafter, it is believed that males have faster growth rates. Several different embryo stages may be recovered at the time of embryo collection at Day 6-8 after estrus. Embryos at the morula stage have fewer cells than blastocysts. Some of the differences in the developmental stages found at embryo recovery may be due to asynchronous ovulations and fertilization of the oocytes, although some differences in growth rate of the embryos may be due to inherent differences in embryo cleavage rates. Avery et al. (1989) found that in the 1/3 of embryos which developed the fastest (most cells) 71% will be male, the middle third will be 50% male and the slowest third will be 20% male. However, it was noted that this tendency is only significant if three different developmental stages are found in the embryo flush, yet only 24% embryo recoveries produce embryos at three developmental stages.

Measurement of X-linked enzymes

Another method to sex preimplantation embryos non-invasively involves assessing X-chromosome linked enzyme activity. The X-chromosome is larger than the Y-chromosome and carries genes which are not found on the Y-chromosome. Therefore an early
embryo with two X-chromosomes (female) will be more likely to express those X-linked gene products than a male embryo with only one X-chromosome. Assessing the metabolic activity of X-linked enzymes has been used to diagnose the sex of embryos. This usually involves a period of culture in a medium containing the substrate for a particular X-linked enzyme, then the conversion rate of the substrate to a coloured or radiolabelled product can be measured. Williams (1976) assayed the activity of glucose-6-phosphate dehydrogenase in mice embryos. Those embryos scored as having two copies of the X-linked enzyme produced 72% females, while those with scored as having one copy produced 57% males. Monk and Handyside (1988) compared X-linked enzyme activity to an autosomal enzyme activity in mice embryos and then sexed the resulting fetuses at d15 of gestation. Fourteen of fifteen fetuses were correctly sexed. Some difficulties exist in measuring X-linked enzyme activities: one is that because females have two X-chromosomes and males only one X-chromosome, females would have excess production of X-linked genes. Nature has compensated for this by having one X-chromosome become inactivated in females. It is unclear at what stage X-inactivation occurs during embryonic development and thus may affect the amount of X-linked genes being expressed. Also, Rieger et al. (1984) had previously tested X-linked enzymes and stated that it was necessary to compare the X-linked enzyme activity to autosomal enzyme activity to standardize for the metabolic rate of individual embryos.
Detection of H-Y antigen

H-Y antigen has been localized on 50% of mammalian embryos after the 8-cell stage (Krcz and Goldberg, 1976). There have been many attempts to detect H-Y antigen on male embryos as a method of embryo sexing. The cytotoxic assay was developed first and involves culturing embryos with H-Y antisera (Krcz and Goldberg, 1975) or monoclonal H-Y antibodies with embryos in the presence of complement for several hours. Affected embryos have lysed cells or are retarded in development and are considered to be males. When unaffected embryos have been karyotyped or transferred to recipients, 80-90% are females (Epstein et al., 1980; White et al., 1983, 1984; Shelton et al., 1984). However cytotoxic assays destroy most of the male embryos, therefore the fluorescent immunoassay for detecting H-Y antigen on embryos has been employed. Embryos are cultured first with anti-H-Y antibodies, washed and cultured in media containing a second antibody (bound to a fluorescent dye) which can bind to the anti-H-Y antibody. Embryos are washed again and then evaluated using a fluorescent microscope. Fluorescent embryos are considered to be males. Seventy-eight to eighty-five percent of the fluorescent embryos have been karyotyped to be males and 83-97% of the nonfluorescent embryos have been karyotyped to be female in cattle (White et al., 1984, 1987). Some difficulties in sexing using the H-Y antibody are H-Y antigen is weak and in cytotoxicity assays some males may not be affected, whereas in immunofluorescent assays dead cells may take up the stain non-specifically. Piedrahita and Anderson (1985) report that the titre of the H-Y antisera affects the accuracy of embryo sexing.
Invasive methods

Detection of the Barr Body

Invasive methods have the advantage of being very accurate, although one or more cells must be removed from the embryo to perform sexing and this may affect embryo viability. One of the earliest methods to diagnose embryonic sex was by the use of Barr body identification. In females, one X-chromosome is inactivated in most cells so that excess X-gene expression does not occur, thus both males and females have one active X-chromosome. The inactivated X-chromosome has been detected as the Barr body, a triangular or ovoid structure in contact with the nuclear membrane in 5 3/4 day old rabbit embryos (Edwards and Garner, 1967). When karyotyped, 41/47 embryos were correctly sexed. However due to the nature of the chromatin, it may not be possible to detect Barr bodies in other species (King, 1984).

Karyotyping

Another method for embryo sexing is to directly visualize the chromosomes to identify the sex chromosomes. Melander (1959) reported that the chromosomal complement in cattle was 2n=60 with all autosomes acrocentric while the X-chromosome is a large metacentric and the Y-chromosome a small metacentric. McFeely (1960) developed a method of preparing karyotypes from Day 10 pig embryos. Whole embryos were cultured for 1h at 37C in colcemid to arrest cells in metaphase, followed by culture in hypotonic solution of one part serum to five parts distilled water. Cells were then fixed, agitated and centrifuged using a fixative of three parts methanol to one part acetic acid in
siliconized glassware, cells were then dropped onto a clean grease-free slide and dried before examination. Initially investigators karyotyped large numbers of cells excised from trophoblasts of expanded d12-18 bovine embryos. Hare et al. (1976) sexed two week old bovine embryos using this method and was able to sex 20/34 embryos. However, the pregnancy rate after transfer of excised embryos was only 37.5%.

Due to the ease of collection, transfer and freezing techniques, most embryo transfer work is now done on d7-8 embryos. Most of the methods used to karyotype zona-enclosed embryos have been developed from the air-drying method (Tarkowsky, 1966). The technique involves exposure of the embryos to colchicine for a few hours to arrest dividing cells in metaphase when the chromosomes are contracted and easy to identify. Embryos are then cultured in a hypotonic solution of 1% sodium citrate to cause swelling of the cells and dispersal of chromosomes in the nucleus, followed by the placement of the embryo on a clean slide and addition of a few drops of 3:1 ethanol-acetic acid to dissolve the zona pellucida and fix the embryo on the slide. The embryo is then stained. Similar methods have been successfully applied to sexing bovine embryos. King et al. (1979) used a solution of 1:1 methanol-acetic acid to soften the zona pellucida prior to 3:1 methanol-acetic acid to fix the embryo with good results. With the low numbers of cells available, it was important to obtain cells with good quality metaphases; the culture time, concentration and time exposed to colchicine was very important. Dyban et al. (1983)
modified Tarkowsky's air-drying method in the culturing embryos with colcemid for 2h, the use of cold 0.9% sodium citrate for 20 minutes to three hours, the use of 1:1 methanol-acetic acid as a zona softening solution and 3:1 methanol-acetic acid as a fixative, followed by Giemsa staining, with good results. However, Murray et al. (1985) found that after 10h culture in 0.8µg/mL colchicine only 40% of the embryos processed had metaphases and in only 25% was the sex analyzable. Picard et al. (1984) used one half of bisected embryos for sexing and after 4h culture in 0.05µg/mL colcemid 60% embryos were sexable. Furthermore, the pregnancy rate from fresh bisected embryos was 60%. Rall and Leibo (1987) obtained an average of 2.64 analyzable metaphases per embryo with 62% sexable after overnight culture of bisected embryos exposed to colcemid in the last 2 hours of culture. Using the method of King (1984), whole or part embryos may be processed for sexing within 5-6 hours.

Although karyotyping is an easy technique to perform, it may be often limited by the quantity and quality of metaphases available for analysis.

Use of probes for Y-chromosomal DNA

DNA probes have been developed which can specifically detect the presence of Y-chromosomal DNA in man (Bishop et al., 1983), mice (Nishioka et al., 1986) and in cattle (Leonard et al., 1987). Leonard et al. (1987) removed 10-20 cells from bovine embryos and used a radiolabelled Y-probe for in-situ
hybridization. The results from sexing were obtained within 30 hours. Bondioli et al. (1989) claimed that in order for a Y-probe to be useful, the probe needs to be a) male-specific and b) detect repetitive DNA on the Y-chromosome, which is particularly important when the number of cells assayed is small. Biopsies of 10-20 cells have been used while the remainder of the d6-7 embryos can be frozen to await the results of the sexing assay and transferred when convenient. After biopsy of Day 6-7 embryos, 90% were sexable at 97% accuracy; biopsied embryos gave a 40% pregnancy rate. Sexing bovine embryos using a Y-probe was also performed on a commercial basis with 41% pregnancy rate and 100% accuracy. Polymerase chain reaction (PCR) is a technique that has been used to amplify DNA probing (Saiki et al., 1985). Sexing embryos with Y probes in conjunction with PCR will take less time to obtain results and may require as little as one cell to be removed from an embryo (Handyside et al., 1989).

Aims and Objectives

1) Two experiments were carried out in the course of the Master’s degree program to attempt to improve superovulatory responses and decrease response variability in dairy cattle. These two experiments are described in chapter 2 and are based on reports that show the presence of a dominant follicle at the time of initiation of superovulation decreases superovulatory response (Guilbault et al., 1991; Huhtinen et al., 1992). The experiments were designed to study the response to superovulation, using ultrasound scanning, at times in the
The first experiment (Chapter 2.1), studied the initiation of superovulation at the beginning of the cycle, before a dominant follicle is evident, in comparison to the traditional mid-luteal treatment. The second experiment (Chapter 2.2) was based on reports that the dominant follicle present on day 7 of the cycle has large numbers of hCG-receptors (Ireland and Roche, 1983) and can be induced to ovulate following administration of 1000i.u. hCG (Rajamahendran and Sianangama, 1992). Superovulatory responses were compared between cows which had the dominant follicle eliminated before superovulation induction and control cows which were superovulated in the mid-luteal phase when a dominant follicle may have been present. Both experiments were carried out to determine whether initiation of superovulation in the absence of a dominant follicle may improve superovulatory responses.

2) In many livestock enterprises, offspring of one sex may be preferred over another. In dairy cattle, females may be preferred for herd replacements while males may have little value except as veal calves. However, superior dairy cows may have large monetary contracts to produce males for artificial insemination organizations. In beef cattle, males may be preferred for their more efficient growth rates. When the costs of superovulation and embryo transfer are high, it may become necessary to ensure the desired sex of calf is born. In chapter 3, three experiments on embryo sexing have been described. Chapter 3.1 discusses the development of H-Y antibodies, and
development of an ELISA to measure anti-H-Y antibody titre. Chapter 3.2 studies the effect of maternal anti-H-Y antibody production on the in-vivo sex ratio. Chapter 3.3 provides a description of bovine embryo karyotyping.
Chapter 2 Experiment One

FOLLICULAR GROWTH, OVULATION AND EMBRYO RECOVERY IN DAIRY COWS GIVEN FSH AT THE BEGINNING OR MIDDLE OF THE ESTROUS CYCLE

Summary

The variability of the superovulation response is an important problem to the embryo transfer industry. The objective of this study was to determine whether FSH treatment at the beginning of the cycle would improve the ovulation rate and embryo yield in dairy cows. Twenty-eight cycling postpartum cows were allocated at random to four treatment groups: A, B, C and D. Control cows (group A) received FSH (35mg) at a decreasing dose starting Day 9-13 of the estrous cycle (D0 = standing estrus) for 5 days and PGF\textsubscript{2\alpha} (25mg) was given on the fourth day of FSH treatment. Cows assigned to treatments B, C and D (n=6 cows, respectively) were given 35mg FSH at a decreasing dose from Days 2 to 6 and PGF\textsubscript{2\alpha} on Day 7. Group C and D cows, in addition, received a progesterone releasing intravaginal device (PRID) from Day 3 to 7. Group D cows also received 1000 i.u. hCG 60h after PGF\textsubscript{2\alpha}. Ovaries were scanned every other day using a real-time ultrasound scanner from the beginning of FSH treatment until the time of embryo recovery to monitor follicular development, ovulations and number of unovulated follicles. Embryos were recovered from the uterus by a non-surgical flushing technique seven days after breeding. There were no differences (p > 0.05) in the number of follicles >10mm 48h after PGF\textsubscript{2\alpha} among treatment groups. The mean number of follicles were 10.6 ± 1.2, 9.3 ± 1.3, 12.2 ± 1.3 and 15.0 ± 2.9 for groups A, B, C and D, respectively. Significantly (p <
more ovulations were observed and embryos recovered in group A cows. Results of this study indicate that treatment with FSH at the beginning of the cycle caused sufficient follicular development but resulted in a very poor ovulation and embryo recovery rates.

**Introduction**

An effective procedure for superovulation of cattle at the present time appears to be administration of gonadotrophin (FSH) during mid-cycle followed by administration of prostaglandin F\(_2a\) (PGF\(_2a\)) 48h later to induce luteal regression, estrus and ovulation (Elsden et al., 1978; Monniaux et al., 1983). However, the variability in response in terms of quantity and quality of the ova produced with the above protocol remains one of the limiting factors in the embryo transfer industry (Monniaux et al., 1983). Variability in response may be caused by extrinsic factors such as drug dosage (Bellows et al., 1969), method of administration (Chupin and Procureur, 1982), and purity of the superovulatory homone preparations (Murphy et al., 1984) as well as intrinsic factors such as ovarian status at the time of treatment (Monniaux et al., 1983). Many studies have attempted to increase follicular development and ovulation in individual cows and to reduce the variability of response between cows but with little success (Goulding et al., 1991; Savio et al., 1991).

Studies using ultrasound imaging in cattle have described estrous cycles with two and three waves of follicular growth
(Pierson and Ginther, 1987; Savio et al., 1988; Sirois and Fortune, 1988; Rajamahendran and Walton, 1988). A wave of follicular growth is characterized by the appearance of a pool of small follicles and the emergence of a single dominant follicle while the remainder of its cohorts regress. A pool of small follicles appears on the day after ovulation, in the middle of the cycle (Days 11-12, Day 0 = day of estrus) and, depending on whether the cycle has two or three waves, at the end of the cycle (Days 15-17). The dominant follicle of the first wave in both two and three wave cycles is identifiable by Day 4, reaches a maximum diameter at Day 6, is static in size Day 6-10 and is not identifiable by Day 15. The dominant follicle of the second wave is identifiable on Day 12 and will ovulate in a two wave cycle while a third dominant follicle will ovulate in a three wave cycle (Savio et al., 1988). Decreases in ovulatory responses have been reported when superovulation has been initiated in the presence of a dominant follicle (Guilbault et al., 1991).

Based on this information, a logical time to begin superovulation treatment to avoid a dominant follicle to improve response and reduce variability may be at the beginning of the cycle. Priming with FSH at the beginning of the cycle and subsequent superovulatory treatment at mid-cycle have been shown to improve response in some studies (Rajamahendran et al., 1987; Ware et al., 1988) but not in others (Rieger et al., 1988). Recently Goulding et al. (1990) reported decreased superovulatory responses and embryo recovery when superovulation
treatments had been initiated at the beginning of the cycle. However, it was not clear from this study whether the decreased response was due to reduced follicular development or inability of the induced follicles to ovulate. The objectives of this study were to (a) monitor ovarian follicular development using ultrasound scanning following FSH treatment at the beginning of the cycle and (b) examine the effect of administering a progesterone releasing intra-vaginal device (PRID) and/or human chorionic gonadotrophin (hCG) along with FSH/PGF$_{2a}$ at the beginning of the cycle on ovulatory response and embryo recovery in dairy cows.

Materials and Methods
Animals

This study was conducted at the University of British Columbia Dairy Unit between September 1989 and March 1991. Holstein cows used in the experiment weighed 450-600 kg and were either lactating or dry. Dry cows were housed in an open lot with a shelter and fed hay. When used for the trial, they were housed in tie-stalls and fed hay or 12 kg alfalfa cubes daily. Lactating cows were cycling and at least 40 days postpartum at initiation of superovulation treatment and were housed in free stalls with limited access to an exercise lot. In addition to alfalfa cubes and hay, they were fed a 16% protein textured dairy ration according to the level of production. Lactating cows were milked twice daily at 0230 and 1430 h. Cows ranged in age from 2 to 9 years.
Treatment

Twenty-eight cows were assigned at random to four treatments A, B, C and D. Cows assigned to treatment A (control n=10) were superovulated starting at day 9-13 (Day 0=estrus) by injecting intramuscularly (i.m.) with a total dose of 35mg porcine FSH (Folltropin, Vetrepharm Inc., Canada) over five days at a decreasing dose of 5.25, 5.25, 4.375, 4.375, 3.5, 3.5, 2.655, 2.655, and 1.75, 1.75mg (8:30a.m. and p.m. daily). A dose of 25mg prostaglandin $F_2\alpha$ (Lutalyse, Upjohn, Canada) was administered i.m. at the time of the seventh injection of FSH. Cows assigned to treatments B (n=6), C (n=6) and D (n=6) were given FSH (35mg divided dose as in the control group) from Days 2 to 6 and $PGF_2\alpha$ (25mg) on Day 7. Treatment C and D cows received an progesterone releasing intra-vaginal device (PRID, Ceva Ltd, USA) from Days 3 to 7. Cows in group D, in addition, received 1000i.u. human chorionic gonadotrophin (hCG) (A.P.L., Ayerst Laboratories, Canada) about 60h after $PGF_2\alpha$.

Ultrasound Examination

The ovaries of the cows were scanned every other day commencing on the first day of FSH treatment and continued until the day of embryo recovery, to monitor ovarian status at the commencement of FSH treatment, follicular growth, ovulations and unovulated follicles. Ovaries were scanned as described previously (Taylor and Rajamahendran, 1991) using a linear array ultrasound scanner (Tokyo Keiki LS300, Tokyo Keiki Co. Ltd., Japan) equipped with a 5MHz rectal probe. Images of the desired structures could be frozen on the screen and hardcopies could be
made with the printer unit.

Estrus Detection, Breeding and Embryo Recovery

Cows were observed twice daily after PGF$_2$ for signs of estrus with the help of Kamar heat detectors (Kamar Inc, Steamboat Springs, Colorado). Cows were inseminated twice, roughly 12 and 24 hours after standing estrus. Thawed semen obtained from the British Columbia Artificial Insemination Centre was used for breeding. Cows with at least one corpus luteum (CL) were flushed seven days after breeding using a non-surgical procedure described earlier (Rajamahendran et al., 1987). The flushings were collected into a sterile collecting bottle, siphoned and filtered to remove excess media. Embryos were located using a dissecting microscope and quality was evaluated using a system described by Reynard and Heyman (1979).

Statistical Analyses

Various parameters measured such as follicular number 48h after PGF$_2$ injection, number of ovulations, number of unovulated follicles, number of ova and transferrable embryos recovered were analysed using the SAS GLM procedure and least square means using Tukey’s, Duncan’s and SNK tests, to determine treatment differences (SAS, 1987).

Results

Typical ultrasonographic pictures of FSH-stimulated follicles 48h post PGF$_2$ treatment, corpora lutea and unovulated follicles observed in cows treated at the beginning of the cycle.
and at the middle of the cycle are shown in Plate 2.1.1 and 2.1.2, respectively. Plate 2.1.1a demonstrates the left (L) and right (R) ovaries of a cow prior to initiation of FSH treatment on Day 2 of the estrous cycle. Small follicles are indicated by arrows. Plate 2.1.1b shows follicular development after FSH treatment. Regressing unovulated follicles, several days after PGF$_{2\alpha}$ injection are indicated by arrows in Plate 2.1.1c. Plate 2.1.2 shows ovarian responses in a cow superovulated at mid-cycle. An arrow indicates the corpus luteum on the left ovary and a dominant follicle on the right ovary in Plate 2.1.2a. Numerous follicles stimulated by FSH treatment are shown in Plate 2.1.2b. Plate 2.1.2c demonstrates multiple corpora lutea in both ovaries at the time of embryo collection. An unovulated follicle is marked by an arrow.

The mean number of follicles >10mm 48h post PGF$_{2\alpha}$, corpora lutea and unovulated follicles at the time of embryo recovery are shown in Figure 2.1. There were no differences (p > 0.05) in the number of follicles >10mm among treatment groups. However, there was a tendency for increased follicular development in cows which received exogenous progesterone, groups C and D. The mean (± s.e.) number of follicles were 10.6 ± 1.2, 9.3 ± 1.3, 12.2 ± 1.3 and 15.0 ± 2.9 for groups A, B, C, and D, respectively. Significantly more corpora lutea (p < 0.005) were observed in group A cows (8.7 ± 1.1) compared to cows superovulated at day 2 (0.3 ± 0.3, 0.0 ± 0.0, and 2.5 ± 2.2 for groups B, C and D, respectively). The number of unovulated follicles at the expected time of embryo collection was
Plate 2.1.1 Follicles, corpora lutea and unovulated follicles in a cow superovulated at day 2 of the estrous cycle.
Plate 2.1.2  Follicles, corpora lutea and unovulated follicles in a cow superovulated at mid-cycle.
Figure 2.1 The mean number (± s.e.) follicles >10mm, corpora lutea and unovulated follicles in cows treated with FSH/PGF$_{2\alpha}$ in the middle (A) or at the beginning of the estrous cycle (B, C, D). Treatment C and D cows received a PRID during FSH treatment. D cows in addition received 1000i.u. hCG 60h after PGF$_{2\alpha}$. Values with different superscripts differ (a,b: $p < 0.001$; c,d $p < 0.01$).
<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of cows exhibiting standing estrus</th>
<th>No. of cows with &gt;2 ovulations</th>
<th>Mean (± s.e.) no. of transferrable embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A: FSH, Days 9-13 PGF$_{2\alpha}$, Day 12 n=10</td>
<td>10</td>
<td>9</td>
<td>4.30 ± 0.66$^a$</td>
</tr>
<tr>
<td>Group B: FSH, Days 2-6 PGF$_{2\alpha}$, Day 7 n=6</td>
<td>1</td>
<td>1</td>
<td>0.17 ± 0.85$^b$</td>
</tr>
<tr>
<td>Group C: FSH, Days 2-6 PRID, Days 3-7 PGF$_{2\alpha}$, Day 7 n=6</td>
<td>0</td>
<td>0</td>
<td>0.00$^b$</td>
</tr>
<tr>
<td>Group D: FSH, Days 2-6 PRID, Days 3-7 PGF$_{2\alpha}$, Day 7 hCG, Day 9 n=6</td>
<td>1</td>
<td>1</td>
<td>1.00 ± 0.85$^b$</td>
</tr>
</tbody>
</table>

$^a,b$ p < 0.05
Values with different superscripts are significantly different. Significantly lower in group A cows (p < 0.01) than groups B, C, and D.

The incidence of standing estrus, numbers of cows with more than two ovulations and the mean numbers of transferrable embryos are shown in Table 2.1. Estrus response was very poor in cows superovulated in the beginning of the cycle, whereas all ten of the cows superovulated in the mid-luteal phase exhibited standing estrus about 48h post PGF$_{2\alpha}$ injection. Nine of ten cows in group A possessed more than two CL and significantly more transferrable embryos were recovered from this group (p < 0.05).

Discussion

A PRID was given to cows in groups C and D to maintain early luteal phase progesterone levels (Rajamahendran et al., 1981) and to prevent premature ovulation of FSH-stimulated follicles in the absence of a functional CL at the beginning of the cycle. PRID treatment did not improve ovulation or embryo recovery rates. Some evidence exists in the literature that progesterone levels at the time of initiation affects the success of superovulation treatments (Yadav et al., 1986; Goto et al., 1987), although other researchers have found no significant relationships (Sreenan and Gosling, 1977; Tamboura et al., 1985). It is unlikely that a single PRID could have had any detrimental effects on stimulated follicles as treatment with a PRID or norgestomet have been used successfully in
superovulation regimens (Kunkel et al., 1979).

The presence of a dominant follicle at the time of initiation of superovulation treatment has been shown to decrease superovulatory response (Guilbault et al., 1991). It has been hypothesized that administration of gonadotrophins at the beginning of the estrous cycle, when there is no dominant follicle to exert its inhibitory effect, would result in increased superovulatory responses and embryo recovery compared to mid-cycle superovulated cows. However, the results observed clearly demonstrate that the ovulatory response and embryo recovery were significantly lower in cows treated at the beginning of the cycle, compared to the mid-luteal phase treated cows. This observation is in agreement with other reports (Sreenan and Gosling, 1977; Lindsell et al., 1985; Goulding et al., 1990) although very few cows treated at the beginning of the study exhibited estrus, ovulated and produced embryos in this study.

Poor superovulatory response and embryo yield in cows treated at the beginning of the cycle was not due to poor follicular development, as the number of ovulatory-sized follicles present 48h after PGF$_2\alpha$ injection, measured using ultrasound, were not different between early or mid-cycle treated cows. Superovulation treatment at the beginning of the cycle, in the absence of a dominant follicle was expected to increase the numbers of follicles generated. However, recent reports indicating high levels of inhibin in small follicles at
the beginning of the cycle (Goulding et al., 1991b) could partly explain the follicular response observed.

Therefore, the poor superovulatory response and embryo yield observed in cows treated at the beginning of the cycle could be attributable to ovulatory failure, as significantly higher numbers of unovulated follicles were present at the normal time of embryo collection in these groups. Inability of the CL to respond to PGF$_2$u, failure of the follicles to secrete sufficient estrogen to cause the pre-ovulatory LH surge, inability of the follicles to respond to LH and atresia of the stimulated follicles are possible causes of failure to ovulate. Based on the ultrasound data, in the majority of cows treated at the beginning of the cycle, the CL was maintained or underwent only partial luteolysis after PGF$_2$u on Day 7. This implies that FSH treatment and subsequent follicular development may have altered the susceptibility of the CL to PGF$_2$u, as administration of PGF$_2$u on Day 7 of the normal estrous cycle causes complete luteolysis (King et al., 1982; Momont et al., 1984; Savio et al., 1990).

Administration of hCG 60h after PGF$_2$u also did not improve ovulation or embryo recovery rates. This implies that large follicles stimulated by FSH administration at the beginning of the cycle (a) did not have sufficient LH receptors, as in previous experiments hCG given on Day 7 of the cycle caused ovulation of large follicles (Rajamahendran and Sianangama, 1992) or (b) were atretic, because large follicles present in
the early luteal phase of the cycle are able to ovulate following PGF$_2\alpha$ administration (Savio et al., 1990). Administration of PMSG on Day 2 or 3 of the sheep estrous cycle has been shown to cause large atretic follicles to develop (Cran, 1983).

In conclusion, the results of this study show that superovulation with FSH at the beginning of the cycle causes sufficient follicular development but results in very poor ovulation and embryo recovery rates. Administration of a PRID or hCG did not improve results obtained from early cycle superovulation attempts. Reduced sensitivity of the CL to PGF$_2\alpha$ and/or abnormal follicular development are possible causes of ovulatory failure in cows superovulated at the beginning of the cycle.
Chapter 2 Experiment Two

SUPEROVULATORY RESPONSES IN DAIRY COWS FOLLOWING OVULATION OF THE DOMINANT FOLLICLE OF THE FIRST WAVE

Summary

This experiment investigated using hCG on Day 7 of the estrous cycle (Day 0=Day of standing oestrus) to ovulate the dominant follicle prior to FSH treatment and the associated increase in progesterone as a method of improving superovulatory response in dairy cows. Twenty cycling lactating cows were allocated at random to two groups each of ten animals, control and hCG-treated. Ovaries of each cow were scanned using ultrasound equipment on Day 7 to confirm the presence of the dominant follicle and thereafter every other day until embryo recovery. All cows received a total dose of 400mg FSH in decreasing amounts for five days (Day 9-13) and 35mg PGF$_{2a}$ on Day 12. Treated cows, in addition, received 1000i.u. hCG on Day 7. Cows were inseminated twice during estrus and embryos were recovered seven days later by a non-surgical procedure. Blood samples were taken several times during the treatment period for progesterone determination. All cows on Day 7 possessed a dominant follicle and all but one of the hCG-treated cows ovulated the dominant follicle and formed an accessory corpus luteum in response to hCG treatment. Plasma progesterone concentrations were not different ($p > 0.05$) between control and hCG-treated cows at Day 7. Plasma progesterone concentrations were significantly higher ($p < 0.01$) on Day 9, the first day of FSH treatment; and on Day 12, the day of PGF$_{2a}$ ($p < 0.005$) in hCG-treated cows. Mean follicular number at estrus, ovulations,
total and transferrable embryos were not different (p > 0.05) between control and hCG-treated cows. However, there was a tendency towards increased superovulatory responses and embryo yield in hCG-treated cows.

Introduction

Superovulation is a method of increasing the number of eggs ovulated by the female. An effective procedure for superovulation in cattle at the present time appears to be administration of follicle stimulating hormone (FSH) during mid-cycle followed by prostaglandin $F_{2\alpha}$ (PGF$_{2\alpha}$) 48h later to induce luteal regression, estrus and ovulation (Elsden et al., 1978; Monniaux et al., 1983). However, the variability in terms of the quality and quantity of embryos produced with the above protocol, remains one of the major limiting factors in the embryo transfer industry (Monniaux et al., 1983). Variability in response may be caused by both extrinsic factors such as drug purity (Murphy et al., 1984), drug dosage (Bellows et al., 1969) and drug administration (Chupin and Procureur, 1982); and intrinsic factors such as ovarian status at the time of treatment (Monniaux et al., 1983).

Studies using ultrasound imaging in heifers and cows have described the estrous cycle as having two or three waves of follicular growth (Roche and Boland, 1991). However, in cows, two waves of follicular growth appear to be the norm (Taylor and Rajamahendran, 1991). A wave is characterized by the appearance of a pool of follicles and the emergence of a single dominant
follicle while the remainder of its cohort regress, probably due to the inhibitory effects of substances produced by the dominant follicle (Ireland and Roche, 1987; Ko et al., 1991). The dominant follicle of the first wave of follicular growth, in cycles with two or three waves, is identifiable by Day 3 (Day 0 = Day of estrus), reaches a maximum diameter on Days 6-7, is in a static phase from Day 6-10 and is not identifiable by Day 15 (Savio et al., 1988). Recently, there have been reports stating that the presence of a dominant follicle at the time of initiation of FSH treatment during mid-cycle reduces the superovulatory response (Guilbault et al., 1991; Huhtinen et al., 1992).

Recently it has been reported that administration of 1000i.u. human chorionic gonadotrophin (hCG) on Day 7 of the estrous cycle results in the ovulation of the dominant follicle, accessory corpus luteum formation and significant increase in plasma and milk progesterone concentrations (Rajamahendran and Sianangama, 1992). Therefore, the objectives of this study were a) to examine the effects of eliminating of the dominant follicle with hCG prior to FSH treatment and b) examining the hCG-induced increase in progesterone concentration on follicular development, ovulations and transferrable embryos recovered in superovulated lactating dairy cows.

Materials and Methods

Animals

Twenty lactating Holstein cows ranging from 2 to 11 years
of age, cycling and at least 40 days postpartum were selected for the study between May 1991 and January 1992 from the University of British Columbia dairy herd. Cows received a mixed ration of alfalfa cubes and a 16% protein dairy textured grain, according to milk production. As well cows were provided with good quality alfalfa hay. Cows were milked twice daily between 0230 and 0500 h and 1430 and 1700 h. Cows were checked for signs of estrus at both milkings and for approximately 1/2h during the evening.

Treatment

Cows were allocated at random to two treatment groups, control (n=10) and hCG-treated (n=10). Cows assigned to the hCG group received 1000i.u. hCG (A.P.L., Ayerst Laboratories, Montreal) on Day 7 of the cycle (Day 0=day of standing estrus), after confirming the presence of the dominant follicle of the first follicular wave with ultrasonographic examination. Both control and hCG-treated cows were superovulated on Days 9 to 13 of the cycle by injecting intramuscularly (i.m.) 400mg Folltropin-V (NIH-FSH-P1, VetrepPharm Inc., Canada) at a decreasing dose of 60; 50; 40; 30 and 20mg b.i.d. at 0830 and 2030 daily. A total of 25mg and 10mg \( \text{PGF}_2 \) (Lutalyse, Upjohn Co., Canada) were administered with the seventh and eighth FSH injections, respectively.

Estrus detection, breeding and embryo recovery

Cows were observed three times daily after \( \text{PGF}_2 \) for signs of estrus and standing estrus with the help of Kamar heat mount
detectors (Kamar Inc, Steamboat Springs, Colorado). Cows were inseminated twice, about 12 and 24 h after standing estrus. Thawed semen was used for inseminations. Cows were flushed 7 days after breeding by a non-surgical procedure described earlier (Rajamahendran et al., 1987). The flushings were collected into a sterilized collecting bottle, siphoned and filtered to remove excess media. Embryos were located using a dissecting microscope and quality was evaluated using a system described by Reynard and Heyman, 1979.

Ultrasound examination

A real-time ultrasound scanning device (Tokyo Keiki LS300, Tokyo Keiki Co., Japan) equipped with a 5MHz rectal probe was used to examine the ovaries as described previously (Taylor and Rajamahendran, 1991). The ovaries of all cows were examined on Day 7 of the cycle and thereafter every other day until embryo recovery to monitor ovarian status at the initiation of FSH treatment, follicular growth following FSH, corpus luteum regression following PGF<sub>2α</sub> and formation of multiple corpora lutea following ovulation of the developed follicles. Desired images of the ovaries could be frozen on the screen, measurements taken using built-in calipers and hardcopies made using a video processing unit (Mitsubishi Electronics Co., Japan).

Blood sample collection and progesterone radioimmunoassay

Blood was collected from a coccygeal blood vessel into heparinized tubes from all cows on Day 7, the first day of FSH
treatment (Day 9), the day of PGF$_{2\alpha}$ (Day 12), at standing estrus following PGF$_{2\alpha}$ and the day of embryo collection. Plasma was separated and stored at -20°C until progesterone analysis. Progesterone was measured using a commercially available solid-phase radioimmunoassay kit, Coat-A-Count (Diagnostics Corp., Los Angeles, CA). Plasma or reference standard (0.1mL) was added to tubes coated with specific antibody. Standards contained 0-40ng/mL progesterone. Buffered $^{125}$I-labelled progesterone (1.0mL) was added to all tubes; tubes were vortexed and incubated for 3h at room temperature. The tubes were then decanted and cleaned with a cotton swab above the 1mL mark to remove excess tracer. The tubes were counted for one minute in a gamma counter (Packard Auto-Gamma 500. Packard instruments, Downer’s Grove, IL). The limit of detection of the assay is 0.05ng/mL. Intra- and inter-assay coefficients of variation were 7.9% and 10.3%. Counts were converted to values in ng/mL using SAS (SAS, 1987). This procedure has been validated for measuring progesterone in bovine plasma (Rajamahendran and Taylor, 1990).

Statistical analyses

Mean numbers of follicles > 10mm at standing estrus, number of ovulations, total and transferrable embryos recovered were analyzed using the GLM proc and mean procedures from SAS (SAS, 1987) to determine differences between treatment groups. Least square means analysis was done using Duncan’s test. Covariate analysis among treatment and progesterone concentrations and superovulatory parameters were performed.
using SAS, 1987 using the following models:

\[ Y_{ij} = \mu + T_i + D9_{ij} + D12_{ij} + E_{ij} \]  
Model 1

\[ Y_{ij} = \mu + T_i + D9_{ij} + D12_{ij} + F_{ij} + E_{ij} \]  
Model 2

\[ Y_{ij} = \mu + T_i + D9_{ij} + D12_{ij} + F_{ij} + CL_{ij} + E_{ij} \]  
Model 3

\[ Y_{ij} = \mu + T_i + D9_{ij} + D12_{ij} + F_{ij} + CL_{ij} + coll_{ij} + E_{ij} \]  
Model 4

\( T_i \) is the effect of treatment, of which there were two, control and hCG. The covariates were \( D9_{ij} \) and \( D12_{ij} \), the progesterone values of the \( i \)th treatment and the \( j \)th cow during superovulatory treatment. \( F_{ij}, CL_{ij} \) and \( coll_{ij} \) were respectively, the number of follicles, corpora lutea and progesterone level at the time of embryo collection in the \( i \)th treatment and \( j \)th cow.

The value \( Y_{ij} \) = superovulatory parameter such as number of corpora lutea, progesterone level at embryo collection, total or transferrable embryos, or % transferrable embryos.

Results

Plates 2.2.1 and 2.2.2 demonstrate the response to superovulation, measured by ultrasound, in a control and hCG-treated cow, respectively. Plate 2.2.1a, demonstrates the ovary at the time of initiation of FSH treatment; the corpus luteum in the left (L) ovary is indicated by a large arrow and a dominant follicle is indicated by a smaller arrow in the right (R) ovary. A follicle stimulated by FSH is shown by an arrow in Plate 2.2.1b. Ovarian responses to FSH treatment at embryo collection, in terms of a corpus luteum (large arrow) in the left ovary and an unovulated follicle (small arrow) in the right ovary, are shown in a control cow in Plate 2.2.1c. Plate 2.2.2a demonstrates the presence of a spontaneous corpus luteum (large
Plate 2.2.1  Superovulatory responses in a control cow superovulated in the mid-luteal phase of the estrous cycle.

A

LEFT OVARY
29X27 MM CL
22 MM FOL
23 MM FOL

B

LEFT OVARY
3 FOLLICLES

C

LEFT OVARY
TWO CL

56
Plate 2.2.2. Superovulatory responses in a cow treated with hCG on Day 7 of the estrous cycle to ovulate the dominant follicle prior to superovulation.
arrow) at Day 7 in the left ovary of an hCG-treated cow. A dominant follicle was located on the right ovary (small arrow). Plate 2.2.2b demonstrates the spontaneous corpus luteum (large arrow) in the left ovary, an hCG-induced corpus luteum in the right ovary (small arrow) and FSH-stimulated follicles (triangles) in both ovaries. Corpora lutea (large arrows) at the time of embryo collection in an hCG-treated cow are shown in Plate 2.2.2c.

All cows on Day 7 possessed a dominant follicle (12 to 20mm in diameter) and nine of ten hCG-treated cows ovulated the dominant follicle and formed an accessory corpus luteum. In the cow which failed to ovulate the dominant follicle following hCG, the dominant follicle persisted until embryo recovery. Following superovulatory treatment with FSH, development of multiple follicles were observed in both control and hCG-treated cows. The mean interval to standing estrus following PGF$_{2\alpha}$ was not different (p > 0.05) between control and hCG-treated cows (47 vs. 53 h). The mean number of follicles > 10mm at estrus; mean numbers of ovulations, total and transferrable embryos recovered at embryo collection were not different (p > 0.05) between control and hCG-treated cows (Table 2.2.1). However, there was a tendency towards improved superovulatory responses in hCG-treated cows.

Mean (± s.e.) plasma progesterone levels for control and hCG-treated cows are presented in Table 2.2.2. Plasma progesterone levels were not significantly different
Table 2.2.1 Mean (± s.e.) numbers of follicles, corpora lutea, total ova and transferrable embryos recovered in control and hCG-treated cows following FSH/PGF2α treatment.

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Follicles ( &gt; 10mm)</th>
<th>Corpora Lutea</th>
<th>Total Ova</th>
<th>Transfer. Embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=10)</td>
<td>10.1 ± 1.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.7 ± 2.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.0 ± 2.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.1 ± 1.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>hCG (n=10)</td>
<td>12.4 ± 1.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.5 ± 1.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.8 ± 2.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.8 ± 1.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values in columns with the same superscripts do not differ (p > 0.05).

Table 2.2.2 Mean (± s.e.) plasma progesterone concentration (ng/mL) in control and hCG-treated cows at different times during superovulatory treatment.

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Day 7</th>
<th>Day 9</th>
<th>Day 12</th>
<th>Estrus</th>
<th>Embryo Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=10)</td>
<td>2.6 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.5 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.0 ± 0.4&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.2 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13.5 ± 4.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>hCG (n=10)</td>
<td>2.6 ± 0.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.5 ± 0.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.1 ± 0.5&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.4 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12.3 ± 2.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values in columns with different superscripts differ.

<sup>b,c</sup> p < 0.01  
<sup>d,e</sup> p < 0.005
(p > 0.05) in control and hCG-treated cows on Day 7, day of estrus or at embryo collection. However, significant differences in progesterone concentrations were observed between hCG-treated cows and control cows on the first day of FSH treatment and on the day of PGF$_2$α injection. Plasma progesterone levels (mean ± s.e.) for control and hCG-treated cows respectively, at the time of initiation of FSH treatment were 3.50 ± 0.33 vs. 5.54 ± 0.55 (p < 0.01) and at PGF$_2$α were 4.04 ± 0.45 vs. 6.13 ± 0.46 (p < 0.005).

Discussion

It has been reported previously that the dominant follicle arising the a pool of follicles suppresses the growth of its cohort and possibly causes atresia (Ireland and Roche, 1987; Ko et al., 1991). Presence of a dominant follicle at the time of initiation of superovulation treatment has been reported to decrease superovulatory responses (Guilbault et al., 1991; Huhtinen et al., 1992) or to have no effect on superovulation (Wilson et al., 1990). In the present study, ovulation of the dominant follicle with hCG prior to superovulation treatment did not significantly improve follicular development, ovulations or numbers of embryos recovered. This implies that intrinsic factors other than the presence of a dominant follicle may be responsible for the variation in superovulatory response. However, possible effects of hCG on small antral follicles should not be ignored, as these may have interfered with the beneficial effect of ovulating the dominant follicle prior to superovulation. If hCG does have
inhibitory effects on the small antral follicle populations in the ovary, then elimination of the dominant follicle by cauterization or ultrasound-guided aspiration before superovulatory treatment may improve the superovulatory response.

In this study, nine of ten cows treated with hCG on Day 7 ovulated the dominant follicle and formed an accessory corpus luteum. This is in agreement with previous findings (Rajamahendran and Sianangama, 1992). Significant increases in plasma progesterone following hCG treatment observed in this experiment are in agreement with previous results (Eduvin and Sequin, 1982; Breuel et al., 1990; Rajamahendran and Sianangama, 1992). The increased progesterone levels could have resulted from hypertrophy of luteal cells in the spontaneous corpus luteum (Veenhuizen et al., 1972; Helmer and Britt, 1986) and from the accessory corpus luteum formation.

In this experiment, in nine of ten cows treated with hCG to remove the dominant follicle prior to superovulation, both the spontaneous and the induced corpus luteum regressed following administration of a luteolytic dose of PGF$_{2\alpha}$ and the time from PGF$_{2\alpha}$ to standing estrus was not different between control and hCG-treated cows. This suggests that the hCG-induced corpus luteum is sensitive to PGF$_{2\alpha}$ as early as four days after it is formed. This finding is in contrast to Rusbridge and Webb (1991) where the interval from PGF$_{2\alpha}$ to estrus was extended in heifers with hCG-induced corpora lutea compared to controls.
Howard and Britt (1990) examined responses of CL to PGF$_{2\alpha}$ 2, 4, or 6 days after estrus (spontaneous CL) and 2, 4, or 6 days after hCG given Day 10 of the estrous cycle (hCG-induced CL). Progesterone declined immediately after PGF$_{2\alpha}$ treatment on day 6 for spontaneous CL or day 2-6 after induction of hCG-induced ovulation at mid-cycle. However, the time from PGF$_{2\alpha}$ injection to estrus was extended in hCG-treated heifers.

There are conflicting reports in the literature about the relationship between plasma progesterone at the initiation of FSH treatment and superovulatory responses. Several studies, (Saumande et al., 1975; Sreenan and Gosling, 1977; Tamboura et al., 1985; Walton and Stubbings, 1986) failed to find any relationship between concentration of progesterone on the first day of FSH treatment and superovulatory response. Others (Yadav et al., 1986; Goto et al., 1987) have reported that progesterone level on the first day of FSH treatment is related to embryo quality. Goto et al. (1988) found significant differences ($p < 0.01$) in CL numbers, total and transferrable embryos between cows which had under 3 ng/mL and over 3 ng/mL progesterone in plasma on the first day of superovulation treatment. In this experiment, progesterone concentrations in plasma were increased on the first day of FSH treatment ($p < 0.01$) and on the day of PGF$_{2\alpha}$ ($p < 0.005$) in hCG-treated cows compared to controls. Covariate analysis of the data showed no significant relationships among treatment or progesterone values on the first day of FSH or at PGF$_{2\alpha}$ and the number of follicles stimulated by superovulation, the number of ovulations, total or
transferrable embryos recovered and the percentage of embryos that were transferrable (p > 0.05). It appears that the level suggested by Goto et al. (1987), 3 ng/ml progesterone in plasma, may be a permissive level for superovulation. Animals with under 3 ng/ml tended to have poorer superovulatory responses.

The number of follicles > 10mm at estrus, detected with ultrasound, had a significant effect on the number of ovulations (p < 0.0001), progesterone level at embryo collection (p < 0.005), and the numbers of total (p < 0.0001) and transferrable embryos recovered (p < 0.05). These high relationships are expected because most follicles over 10mm in size will ovulate and release ova capable of being fertilized following the LH surge. The number of follicles stimulated by FSH treatment explained most of the variation in superovulatory response. For example, the first model compared treatment and progesterone concentrations on the first day of FSH and day of PGF$_2$$_a$ and explained only a small amount of variation in CL numbers $r^2=0.091718$. The inclusion of follicle number in second model explained much more of the variation in response, $r^2=0.781896$. Similarly, the second model increased the amount of variability explained for progesterone values at collection, and total and transferrable embryos recovered. However, the number of follicles generated by superovulation has little effect on the proportion of transferrable embryos (p > 0.05, $r^2=0.128063$). As follicle numbers increase, CL numbers and total and transferrable embryos usually increase. The number of follicles developed limits the number of transferrable embryos obtainable
from a donor.

Inclusion of CL numbers, as detected with ultrasound at the time of embryo collection, into the third model explained additional variation in superovulatory responses. CL numbers had significant relationship with total embryos (p < 0.05) and progesterone levels at embryo collection (p < 0.001). CL numbers tended to have a relationship with transferrable embryos recovered (p < 0.10). Numbers of CL also had a significant relationship with the proportion of embryos recovered which were transferrable (p < 0.05).

Inclusion of the plasma progesterone values at embryo collection into the fourth model also helped to explain some variability in response. Plasma progesterone concentration at the time of embryo collection had a significant relationship with transferrable embryos (p < 0.05). However, plasma progesterone at embryo collection was not significantly related with the proportion of transferrable embryos (p > 0.05).

In conclusion, the results of this study demonstrated that elimination of the dominant follicle with hCG prior to superovulation did not improve superovulatory responses or decrease response variability. Progesterone levels were increased during FSH treatment in hCG-treated cows, however this did not have significant effects on superovulatory parameters. The number of follicles generated during superovulation...
treatment was the best predictor of the subsequent ovulatory and collection responses, however the number of follicles generated during treatment did not affect the quality of embryos recovered. Follicles, CL numbers and progesterone levels at embryo collection can help predict the numbers of embryos recoverable from a donor, and can influence decisions about whether it is worthwhile to breed or attempt embryo collection from a donor. However, it would be beneficial to have a marker to predict or increase response before superovulation is actually carried out. It has been postulated that better superovulatory responses are obtained when superovulation is initiated at mid-cycle when a dominant follicle may not be present (Goulding et al., 1990). Because estrous cycles may have two or three follicular waves (Roche and Boland, 1991), and the timing of the window between periods of follicular dominance may not be easily identifiable without the use of ultrasound; it would be useful to investigate other methods to initiate superovulation in the absence of a dominant follicle. Although removal of the dominant follicle with hCG prior to superovulation tended to increase the response; the experiment may have been confounded by actions of hCG on small follicles in the ovary.
Chapter 3 Experiment One

**IMMUNIZATION OF FEMALE MICE AGAINST MALE SPLENIC CELLS AND DETERMINATION OF ANTIBODY TITRE AGAINST H-Y ANTIGEN**

**Summary**

C57BL\6 female mice were immunized against H-Y antigen through injections of thirty million male mouse splenic cells and Freund's adjuvant weekly for six weeks. Blood was collected from the retro-orbital sinus or tail vein and serum was collected to determine H-Y antiserum titre. Two ELISAs were developed to measure H-Y antisera titre (male spleen cell-based and Daudi cell culture supernatant-based). However, large amounts of non-specific antibody cross-reactivity were noted, such that it was not possible to accurately determine specific H-Y antibody titre.

**Introduction**

Both sperm (Goldberg et al., 1971) and male embryos (Krco and Goldberg, 1976) are known to possess H-Y antigen. Many experiments have been designed in the past to attempt to change the sex ratio of offspring to be born. H-Y antiserum, raised in male spleen-immunized C57BL/6 female mice, has been used in many experiments and can be used to kill male pre-implantation embryos in cytolytic assays (White et al., 1983). As well, H-Y antisera has been used to affect the sex ratio after artificial insemination (Bennett and Boyse, 1973; Zavos, 1983) after in vitro fertilization (Hoppe and Koo, 1984) and after breeding of H-Y-immunized females (McLaren, 1962; Lappé and Schalk, 1971; Shalev et al., 1980; Rao et al., 1981); with inconsistent
effects on the sex ratio of litters.

H-Y antibody titre has previously been assayed in cytotoxicity assays (Goldberg et al., 1971) and in enzyme-linked immunosorbent assays (ELISA) (Brunner et al., 1984, 1988; Iyer et al., 1989).

An experiment was designed to investigate immunization of C57BL/6 female mice against male splenic cells to raise H-Y antisera and to develop an ELISA system to measure H-Y antiserum titre.

Materials and Methods
Immunization Procedure and Sera Collection

Virgin C57BL/6 (B6) male and female mice were originally obtained from Charles River. Thereafter, a colony of B6 mice was established. Six to eight week old females were immunized at weekly intervals for six weeks against male B6 spleens. Males were euthanized with CO$_2$ gas, abdomens opened using sterile dissecting instruments and spleens removed aseptically and placed into a 35mm petri dish in Dulbecco’s phosphate buffered saline (PBS). The PBS was injected into several sites in each spleen using a 1mL syringe and 26g needle, until the spleen turned pale and the PBS became quite red. Thus a single cell suspension of splenocytes was made. Dislodged splenocytes were pooled and centrifuged at 1000g for 5 minutes. The cell pellet was resuspended in PBS and an aliquot was counted on a hemocytometer. The suspension was then made up to contain 300
million splenocytes/mL in PBS. Emulsions were made with complete Freund's adjuvant (CFA) in the first week of immunization and incomplete Freund’s adjuvant (IFA) thereafter. A 1:1 concentration of splenocytes in PBS and adjuvant was made and emulsification was accomplished using a sterile glass syringe in a sterile glass beaker. Male-immunized females were injected with splenocyte/adjuvant mixture containing approximately 30 million cells, subcutaneously in the abdomen in four sites, for a total volume of 0.2mL. Control immunized females received 0.2mL 1:1 PBS and CFA in first week and PBS and IFA thereafter.

Blood collection

Blood was collected from anaesthetized (sodium pentobarbitol i.p. or halothane inhalation) mice from the retro-orbital plexus or by cutting off a small piece of tail with a scalpel blade and bleeding into heparinized microcapillary tubes. Blood was placed into labelled 1.5mL microcentrifuge tubes and centrifuged at 1000g for 5 minutes. Sera was collected and placed into fresh microcentrifuge tubes. Serum samples were heat-inactivated at 56C in a water bath for 30 minutes and then stored at -20C until assayed for anti-H-Y titre. In addition, some control females were bled from the tail vein prior to adjuvant immunization and some control females and males were euthanized by CO₂ gas and blood was collected directly from the opened chest cavity.
Titre determination

Daudi Burkitt lymphoma is a cancer cell which lacks B-microglobulin and thus can not bind H-Y antigen. Daudi cell culture contains a high concentration of free H-Y antigen (Beutler et al., 1978). Daudi Burkitt lymphoma (CCL 213) was obtained from American Type Culture Collection, Maryland. Daudi cells were cultured in RPMI containing 20% fetal calf serum in 5% CO$_2$ at 37C in tissue culture flasks under aseptic conditions in Dr. Lee's Andrology laboratory, U.B.C. Hospital. Every other day, culture supernatant was placed into sterile 50mL Falcon tubes and centrifuged for 10 minutes. The supernatant was discarded, media replaced and cell density checked. When large numbers of cells were present, cells were washed and resuspended in serum-free RPMI and cultured overnight in 5% CO$_2$ at 37C in 24-well Nunc dishes. The supernatant was pooled and stored at -20C until used for an assay. An H-Y monoclonal antibody hybridoma culture (HY3-11.27, HB8116) was also obtained from ATCC for use as a positive H-Y antibody control for ELISAs. It was grown in RPMI and 10% fetal calf serum under 5% CO$_2$ in air at 37C, media was replaced every other day and supernatant collected and stored at -20C until use.

Development of ELISAs to measure anti-H-Y antigen titre

Several preliminary experiments were carried out to determine the proper concentrations of Daudi supernatant (H-Y antigen), monoclonal antibody and sera for use in developing an ELISA. Daudi supernatant was diluted in carbonate- bicarbonate buffer, pH 9.6 (1.59 g Na$_2$CO$_3$ and 2.93g NaHCO$_3$ in 1L distilled
water) at 1-1/20 dilutions, which was expected to produce approximate concentrations of 0.5-10µg/mL protein per well. One hundred microlitres diluted Daudi was added to Falcon 96-well round-bottom plates and held at 4°C overnight. Plates were then blocked with 200µL blocking solution containing 0.1M tris(hydroxymethyl)amino-methane [0.1M Tris-HCl], 0.15M NaCl, 2% sucrose, 0.1% thimerosal and 0.5% bovine serum albumin (BSA) for 1h at 37°C. Plates were then washed three times with PBS and 0.05% tween [polyoxyethylene sorbitan monolaureate] and three times with distilled water, which constituted the standard plate washing regime. Monoclonal antibody was diluted in PBS with 0.5% BSA and 0.1% thimerosal (PBS-BSA) and tested at 1-1/20,000 dilutions. Positive and negative sera were initially tested at 1/50-1/200 dilutions. One hundred microlitres of the mouse sera or monoclonal antibody was added to blocked plates and incubated initially at 37°C for 1h, followed by the standard wash. Next 100µL of the secondary antibody, goat anti-mouse F’c-alkaline phosphatase conjugate (Helix Biotech, Richmond, B.C.) diluted 1/1000 in PBS-BSA was added and incubated for 1h at 37°C, followed by the standard wash. Then 100µL of the alkaline phosphatase substrate containing 2.6mg/mL p-nitrophenyl phosphate in 1.0M diethanolamine buffer pH 10 was added and incubated in the dark at 37°C until adequate colour development. Optical density (O.D.) of the plates were read at various times during colour development at 405nm using an ELISA plate reader. The H-Y antibody hybridoma culture never showed evidence of colour development at any of the dilutions tested. Even after 10x concentration with (NH₄)₂SO₄ and assay with an antibody typing
kit, little or no IgM anti-H-Y activity was noted, and thereafter the supernatant was not used as a positive control. The assay was then designed to directly compare O.D. from sera obtained from male-immunized and adjuvant-only immunized females.

Based on preliminary results, Daudi supernatant was used at 1/2 dilution in carbonate-bicarbonate pH 9.6 buffer and left to coat plates overnight at 4C. Plates were blocked and washed as above. One hundred microlitres H-Y immunized and control mouse sera were tested in duplicate at 1/200 and 1/800 dilutions in PBS-BSA, and was incubated for 3h at 37h. Male mouse sera was similarly diluted and served as a background for antibody binding. Another negative control using PBS alone in place of the mouse sera was done in order to measure the amount of non-specific secondary antibody binding to Daudi-coated wells. Following the standard wash, 100µl goat anti-mouse F'c-alkaline phosphatase diluted 1/1000 in PBS-BSA was added and incubated for 1h at 37C. Plates were washed and 100µL substrate was added and plates were incubated in the dark. Plates were read at 15, 30 and 45 minutes. All of the early samples were assayed in one day. O.D. results were analyzed using SAS, 1987 (covariate analysis).

Sera from the final bleeding of H-Y-immunized and adjuvant-immunized females of the third replication, male controls, parous female and control females before and after adjuvant immunization were assayed using two methods. The first
method was as above using Daudi cells. The second method was by a procedure similar to Iyer et al. (1989). One B6 male was euthanized using CO₂ gas, and the spleen aseptically removed. The spleen was then dissociated by pushing it through a wire mesh in PBS using a 5mL syringe. The concentration of splenocytes was counted using a hemocytometer and cell counts adjusted to 10 million cells/mL using PBS. Fifty thousand male spleen cells in 50μL were added to each well of 96-well cell culture plates and left to dry at 37°C overnight. The splenocyte cells were fixed to the wells by the addition of 100μL 0.5% glutaraldehyde at room temperature for 10 minutes. Plates were washed using the standard wash. Sera was initially tested at 1/100 and 1/400 in PBS-BSA for 3h at 37°C and secondary antibody at 1/1000 in PBS-BSA for 1h at 37°C, but little effect of dilution was noted. Thereafter, 1/200 and 1/800 dilutions of sera and 1/2000 of the secondary antibody were used. One hundred microlitres of 2.6mg/mL p-nitrophenyl phosphate in 1.0M diethanolamine buffer pH 10 was added and incubated in the dark. Optical density of the wells was recorded at 405nm on the ELISA plate reader after 45 minutes incubation with the enzyme substrate.

Results

Overall the results were variable and there were several unexpected findings. However, all dilutions of serum followed expected patterns, i.e. serum diluted at 1:200 always had a higher reaction than serum at 1:800. Male serum was used as a negative control in this assay, because males were not expected
to have antibodies to H-Y antigen. Despite this, some male serum samples exhibited higher reactivity in the ELISA system than some female serum samples. As well, any female exposure to adjuvant tended to increase reaction in the ELISA. One female which had littered several times, yet had not been immunized or exposed to adjuvant, had a relatively high reaction in the assay system. Control females which had been immunized against adjuvant alone (female controls) and had also been bred several times displayed, at various times, H-Y antibody titres (as measured by both Daudi and spleen cell based ELISA systems) higher than H-Y immunized females. The two assay systems tested produced similar results.

Discussion

Serum titres of immunized mice could not be accurately determined in this experiment. The ELISAs developed did measure some antibody reactions, however these seemed to be nonspecific. Male serum was used as a negative control, because males were not expected to have antibodies to H-Y (a self) antigen. Despite this, some male serum samples exhibited higher reactivity in the ELISA system than some female serum samples. As well, any female exposure to adjuvant tended to increase reaction in the ELISA. Overall, this indicates that the ELISA system may not have measured H-Y antibody titres, although all dilutions of serum followed expected patterns and were always higher than PBS alone. The fact that two different assay systems were tested and produced similar results, may indicate that there is a high level of non-specific antibody cross-
reactivity in these assay systems. This has also been reported by Savikurki et al. (1983).

One female which had littered, but had not been immunized or exposed to adjuvant, had a relatively high reaction in the assay system. Control females, immunized against adjuvant alone, had also been bred several times. They exhibited H-Y antibody titres higher than H-Y immunized females. These results may be explained by the fact that females with successive pregnancies (repeated exposure to H-Y antigen on sperm and/or male embryos) may generate H-Y antibodies (Krupen-Brown and Wachtel, 1979).

Male spleens have been used in many previous experiments to generate H-Y antisera (Wachtel et al., 1975; White et al, 1984), yet it is known that male spleen is not a pure source of H-Y antigen. Doubtless, some antibodies react in the ELISA systems, yet it is not known how much binding was due to: non-specific cross-reactive antibodies; adjuvant-stimulated non-specific cross-reactive antibodies; H-Y antigen specific reactivity generated by breeding and pregnancy; and H-Y specific reactivity stimulated by immunization to male spleens.

Overall it appears difficult to obtain specific high titre H-Y antibody in mice (Savikurki et al., 1983). This may be due to several factors: a) H-Y antigen is a weak antigenic stimulus b) only a few strains of mice respond to H-Y antigen c) the accuracy of tests measuring anti-H-Y titre is confounded by the
presence of non-specific antibodies.
Chapter 3 Experiment Two

EFFECT OF MATERNAL IMMUNIZATION AGAINST H-Y ANTIGEN ON LITTER SIZE AND SEX RATIO

Summary

Because H-Y antigen is known to be present on spermatozoa (Goldberg et al., 1971) and male embryos (Krco and Goldberg, 1976); an experiment was designed to test the effect of maternal H-Y antibody production on litter sizes and sex ratios of immunized females. Overall, no effects of treatment, breeding number, sire of litter, replication or treatment by breeding interactions were found by covariate analysis. However, least square means analysis showed a significant difference in the proportion of males born in the first litters of control and H-Y immunized females (p < 0.05). H-Y immunized females had more males in the first litter. This may be related to a beneficial maternal reaction to the foreign H-Y antigen carried by male fetuses, such that male fetuses were advantaged at the time of implantation.

Materials and Methods

Breeding

After the first bleeding following the sixth week of immunizations, described earlier, females were paired singly with CD-1 or B6 males to allow mating. CD-1 males were used to study whether males which differed from the females in antigens other than H-Y antigen, would produce altered litter sizes or sex ratios. Females remained in the male’s cage for two weeks. Females were then placed into individual cages to litter.
Litters were examined within 48 hours of birth and were initially sexed at this time. Final sexing was accomplished at weaning at 21 days. Control and male-immunized females were not immunized during breeding, pregnancy or lactation periods. After the first litter was weaned, females were immunized approximately every two weeks. Sera was collected as above at various times during the experiment.

Litter size and proportions of males born in a litter were examined through covariate analysis using SAS (SAS, 1987) and use of Duncan's test to study least square mean separation. The model used for proportion of males was:

Model 1 \[ Y_{ijkl} = \mu + T_i + R_j + B_k + M_l + (TB)_{ik} + OD + E_{ijkl} \]

Where \( T_i \) is the ith (1-2, control or male-immunized) treatment, \( R_j \) is the jth replication (1-3), \( B_k \) the kth breeding (1-4), \( M_l \) the lth male (1-2, CD-1 or C57BL/6) used for breeding. \( TB \) is the interaction between treatment and breeding, and \( OD \) is the ELISA optical density reading. \( Y_{ijkl} \) = the number of males born in a litter. A similar model was used for litter size, with \( Y_{ijk} = \) litter size:

Model 2 \[ Y_{ijk} = \mu + T_i + R_j + B_k + (TB)_{ik} + E_{ijk} \]

Optical density and breed of male were excluded from this model because they had insignificant effects on litter size.

Results

No significant effects of treatment, replication, breeding, sire or optical density were noted on the arcsine-transformed proportion of males in the litters. Proportions of males in
litters appeared normally distributed and further analyses were done using untransformed male proportions per litter. Overall, model 1 explained very little of the variation in the numbers of males born per litter, \( r^2 = 0.117722 \). Main effects of treatment, replication, breeding, sire and the interaction of breeding and treatment were not significant. However, the overall proportion of males in first litters born to immunized females was 0.61 while it was 0.49 in control females. Least square means analysis demonstrated that the proportion of males in first litters of control and immunized females were significantly different \((p < 0.05)\). Table 3.2.1 demonstrates litter size and sex ratio of the first litter of control and H-Y immunized females in the first replication. The third replication also had an excess of males in the first litter, but litter sex ratio was 1:1 in the second replication.

Litter size was not affected by (anti-H-Y) ELISA O.D. value, sire or immunization treatment. The breeding number affected litter size \((p < 0.05)\). Litter size in the fourth litter was low, however, few females produced four litters.

Discussion

Previous studies using antibodies against H-Y antigen to affect the sex ratio have given differing effects. H-Y antigen is known to be present on spermatozoa \((\text{Goldberg et al., 1971})\). This lead to development of experiments to test the effects of anti-H-Y antisera on fertility. Bennett and Boyse \((1973)\) combined mouse spermatozoa with H-Y antisera and complement
Figure 3.2.1 The number of males and females in the first litter of control and H-Y immunized females in replication 1.

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<td>86</td>
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Mean 3.8 3.4 7.2 Mean 4.6 1.4 6.0
S.D. 1.9 1.5 1.6 S.D. 2.6 1.5 1.6

C cannibalized litter
in a cytolytic assay prior to use for artificial insemination and saw an increase in the numbers of females born. Similarly, Zavos (1983) instilled H-Y antisera intravaginally in rabbits prior to artificial insemination and also showed an increase in the proportion of females born compared to controls. Hoppe and Koo (1984) reacted mouse sperm with monoclonal H-Y antibodies in a sedimentation assay with protein A-sheep red blood cells. Unsedimented (less H-Y antigen) sperm were then used for in vitro fertilization of mouse eggs. Fertilized eggs were then transferred to pseudopregnant recipients and no effect on sex ratio was seen. Since female mouse response to H-Y antigen is known to vary with strain (Eichwald and Silmser, 1955); different strains of mice for use in antisera production, egg and sperm donors, and recipients may have affected the numbers of males born in the previous experiments. Krackow and Gruber (1990) have shown that sex ratio of the litter depends on the strain of mice and mode of conception (matings postpartum or after lactational anoestrus). Postpartum matings in some strains, resulted in larger numbers of females born.

Goldberg et al. (1971) saw no reason to suppose that Y-chromosome bearing sperm should carry more H-Y antigen. The amount of H-Y antigen present is believed to depend on the maturational state of the sperm. H-Y antigen is found on diploid spermatozoa precursors in the testis, but the concentration of H-Y antigen bound to sperm decreases as sperm move through the epididymis (Hoppe and Koo, 1984). Therefore,
it appears that H-Y antigen expression is highest on immature sperm and is not related to whether the sperm is X- or Y-chromosome bearing.

H-Y antigen is found on sperm (Goldberg et al., 1971) and male embryos after the eight-cell stage (Krco and Goldberg, 1976). It was believed at the initiation of this project, that maternal response to H-Y antigen would affect the number of viable sperm or would harm male embryos around the implantation stage. This would be reflected in decreased litter sizes or fewer males born. The results demonstrate that immunization against H-Y antigen does not affect litter size and may provide evidence that sperm are normally inaccessible to attack in the female tract. Alternately, due to large numbers of sperm available, enough sperm may survive immunological attack to provide normal rates of fertilization. The lack of change in litter size in immunized females agrees with previous reports (McLaren, 1962; Rao et al., 1981).

Covariate analysis of breedings of male spleen-immunized females and control adjuvant-immunized females in this experiment indicate that there were increased numbers of males born in the first litter of male-immunized females (p < 0.05). Previously, reported effects of H-Y immunization on sex ratios of litters of immunized females has been controversial. Evidence exists that maternal H-Y immunization prior to breeding caused female-biased litters (Pechan, 1985; Singh and Verma, 1987), no change in the sex ratio (McLaren 1962, Rao et al.,
or male-biased litters in splenectomized and H-Y immunized females (Lappe and Schalk, 1971; Shalev et al., 1980; Hings and Billingham, 1984). The increase in the number of males in the first litter of intact H-Y immunized females in this experiment was an unexpected result.

The increase of males born in the first litter to immunized females, may have been due to a beneficial reaction in the H-Y immunized mother that increased the number of males developing, while maintaining litter size. Previous studies have shown that allogeneic pregnancies are routinely accepted by the mother, despite the presence of foreign antigens on the fetuses. Pregnancy has been shown to increase the number of cells in maternal lymph nodes which drain the uterus, and this effect is more marked in allogeneic pregnancies (Beer et al., 1975). Presensitization to foreign paternal antigens improved, not decreased, reproductive performance (Beer et al., 1975). Lymph node cells may be part of an important maternal reaction which increases angiogenesis at implantation sites. Athanassakis et al. (1987) found that maternal T-cell lymphokines stimulate placental growth.

Lappé and Schalk (1971) found 30% more eggs are ovulated than embryos which implant in mice. Therefore, if 10 eggs ovulate, only 7 embryos will implant. At fertilization, a 1:1 sex ratio would result in 5 male and 5 female embryos. However, if males were favoured at implantation, then 5 males may implant but then only 2 of the female embryos could implant, due to
space and other constraints in the uterus. Females immunized to H-Y antigen in this study may have reacted to the H-Y antigen carried on male embryos and thus improved male implantation rate, thereby increasing the sex ratio.

The overall effect of immunizing females to H-Y antigen is weak and the effect is only noted in the first litter. It has been claimed that the response to H-Y antigen depends on the strength of the antisera (Piedrahita and Anderson, 1985) and that it is relatively easy to induce tolerance to the H-Y antigen (McLaren, 1962; Piedrahita and Anderson, 1985). Therefore, it may well be that an immunized female’s response to male embryos carrying the H-Y antigen depends on the strength of maternal immunization. This is in line with the theories of (Krupen-Brown and Wachtel, 1979; Bell and Billington, 1980) that indicate that successive pregnancies may induce tolerance to H-Y antigen and production of non-complement fixing H-Y antibodies. Prehn (1960) noted that C57BL/6 females which had borne several litters were tolerant to male skin grafts. Either tolerance to H-Y antigen or production of non-complement fixing H-Y antibodies may indicate that the females are no longer able to react to H-Y antigen. Thus any advantage that male fetuses gained in the first pregnancy due to maternal immunization may be lost in successive pregnancies. Further evidence shows that splenectomized-immunized females, which normally had an excess of male progeny, had normal sex ratios if they were re-immunized (Shalev et al., 1980).
Although tests have been developed which can sex embryos with 80% accuracy in vitro (White et al., 1983, 1987); results from experiments carried out partially or wholly in vivo produce inconsistent shifts in sex ratio. This is the first experiment which reported increases in males born to H-Y immunized females, however the effect was not seen in later pregnancies possibly due to induction of tolerance to H-Y antigen through repeated pregnancies (Krupen-Brown and Wachtel, 1979) or through repeated immunizations (Shalev et al., 1980). In conclusion, it does not appear that maternal immunization to H-Y antigen will be able to affect large departures from the 1:1 sex ratio.
SEXING OF BOVINE PREIMPLANTATION EMBRYOS BY KARYOTYPE ANALYSIS

Summary

Due to the fact that sex selection of semen is unreliable and detection of fetal sex at mid-gestation is inefficient, most of the emphasis for obtaining offspring of the desired sex has focussed on sex determination of pre-implantation embryos prior to embryo transfer. Sexing bovine embryos by karyotype analysis is relatively easy due to the ease in identifying the X- and Y-chromosomes. Embryos may be processed and sexed within about 6h, which allows embryos to be transferred fresh or frozen. Although accuracy of sexing embryos is quite high, sexing rate is only 60% due to embryonic and technical difficulties.

Introduction

There are two main methods of sex selection: non-invasive techniques which generally do not affect embryo viability, and invasive techniques which although more accurate may have some detrimental effects on embryo viability. Because phenotypic sex usually agrees with genotypic sex, embryos may be sexed by direct identification of sex chromosomes. Melander reported in 1959 that the chromosomal complement of cattle was 2n=60 with 58 acrocentric autosomes and a large metacentric X-chromosome and small metacentric Y-chromosome. Because X- and Y-chromosomes are easily identifiable, it is possible to determine embryonic sex by karyotype analysis.

Early experiments used large pieces of hatched embryos for
karyotype analysis (McFeely in 1960, Hare et al., 1976). Hare et al. (1976) prepared chromosomes from relatively large pieces of trophoblast from day 14 bovine embryos, and was able to sex 20/34 embryos. After transfer of the biopsied embryos a 37.5% pregnancy rate resulted.

Due to the ease of collection and ability to freeze embryos, most work with embryo transfer procedures is done with Day 6-8 embryos. Correspondingly, most karyotype analysis is also done on the morula/blastocyst stage bovine embryo. Tarkowsky (1966) first used the air-dry method to prepare chromosomal spreads from zona-enclosed mouse embryos. Whole mouse embryos were exposed to colcemid for 1-2h, 1% citrate for 5-15 minutes at room temperature and then were fixed with 3:1 ethanol-acetic acid while blowing air onto the specimen. King et al. (1979) used 1:1 methanol-acetic acid, followed by fixation in 3:1 methanol-acetic acid and was able to analyze 18/20 bovine embryos. Some workers have bisected embryos to form demi-embryos and cultured these for four to ten hours before processing for karyotype analysis to find that 60% were sexable (Picard et al., 1984; Rall and Leibo, 1987). Transfer of the demi-embryos gave 50-60% pregnancy rates (Picard et al., 1984; Rall and Leibo, 1987). However, Murray et al. (1985) were only able to sex 40% of processed embryos after a ten hour culture. Hare et al. (1980) found that the limitations on ability to karyotype embryos were mainly due to the small number of cells available.
This experiment was carried out to learn the techniques of embryo karyotyping as a method of preimplantation sex determination. Analysis of the bovine karyotype can be used as a primary method for sex determination or for confirming the sex of embryos sexed using other methods.

Materials and Methods

First culture of bovine lymphocytes was done in order to have a large numbers of cells to process and gain familiarity with the bovine karyotype. Blood was collected from coccygeal bleeding from cows or male calves into heparinized test tubes. Blood was cultured to produce lymphocyte chromosomal spreads according to the method of Eldridge (1985). Four hundred microlitres of whole blood was placed into 4mL Ham's F-10 media with 0.5mL fetal calf serum and 1 µg/mL phytohemagglutinin-M. This was cultured for 2-3 days at 37°C in 5% CO₂. Then the culture was poured into a graduated test tube and 0.067M potassium chloride was added to make 8mL. This stayed at room temperature for 30 minutes. Then 1mL of 3:1 mixture of methanol/glacial acetic acid was added and the tubes centrifuged at 1000g for 5 minutes. The supernatant was removed and the pellet resuspended in 5mL 3:1 methanol-acetic acid and centrifuged at 1000g for 5 minutes. This process was repeated with three washes with 3mL methanol-acetic acid. Then the pellet was resuspended in 1mL methanol-acetic acid. A pasteur pipette was used to drop a few drops of the fixed cells onto a clean slide from a height of about 20cm. The slide was allowed to dry and was stained for 5 minutes with 4% Giemsa.
After enough experience had been gained with lymphocytes, good quality bovine embryos obtained from the two experiments on superovulation were karyotyped. Embryos were cultured in 35mm petri dishes containing 2mL Ham's F-10 with 10% serum (fetal calf or estrous cow) for 4-5h at 37°C in 5% CO₂ in air, essentially according to King (1984). Colchicine (0.5μg/mL) was used instead of 0.05μg/mL colcemid. After culture, embryos were placed into a 35mm petri dish containing 0.9-1.0% tri-sodium citrate for 5-20 minutes. Embryos were then placed onto a clean grease-free slide in a small amount of the hypotonic solution. A few drops of 4:3:1 (methanol-distilled water-acetic acid) were added to soften and digest the zona pellucida. Then a few drops of 1:1 methanol-acetic acid were placed on the embryos and the slides dried at room temperature. The fixation process was observed under a dissection microscope at 50x. Slides were fixed overnight in a Copplin jar with 3:1 methanol-acetic acid. Slides were dried before staining with 4% Giemsa. Photomicrographs of embryos were taken using oil immersion at 1000x.

Results

Plate 3.3.1 and 3.3.2 show chromosomal spreads prepared from female and male lymphocytes respectively. The large metacentric X-chromosomes are indicated by arrows in Plate 3.3.1. In the male karyotype on Plate 3.3.2, the large X-chromosome (large arrow) and the small metacentric Y-chromosome (small arrow) are indicated. Plate 3.3.3 is a photomicrograph of chromosomes prepared from a bovine embryo. One X-chromosome
Plate 3.3.2 Photomicrograph (1000x) of lymphocytes demonstrating the male bovine karyotype.
Plate 3.3.3. Photomicrograph of chromosomes prepared from a bovine embryo (1000x).
can be easily identified (arrow). There are no other X-chromosomes visible, but due to chromosomal morphology it is difficult to identify the Y-chromosome, although it is likely to be one of the smaller chromosomes. This embryo is tentatively sexed as male.

Of approximately 50 morula/blastocyst bovine embryos processed for karyotyping, only about 15 had good chromosomal spreads. Embryos had from 0-6 metaphases. After staining many of the chromosomes appeared to have indistinct morphology.

Discussion

Karyotype analysis of bovine embryos has been used as a reliable method for embryo sexing and will allow the selection of the sex of the calf before embryo transfer. The technique is highly accurate due to the ease in identification of the sex chromosomes in cattle.

In this experiment using whole embryos, only about 25% of the embryos processed had good metaphase chromosomal spreads. Some difficulties in obtaining good quality metaphases for sexing have been reported. Some of the difficulties experienced in this experiment were: 1) limited number of cells available for sexing 2) lack of metaphases 3) poor spreading due to incomplete digestion of the zona pellucida before fixing 4) poor morphology of the chromosomes 5) incomplete chromosomal spreads and 6) lost embryos. The major difficulty in the course of this experiment was in achieving complete removal of the zona
pellucida. When the zona pellucida was not completely removed before fixation, the embryo would not spread on the slide and even if metaphases were visible, the chromosomes could not be seen clearly due to overlapping in the zona-constrained space. Most authors use micromanipulators to cut the embryo into demi-embryos or to take small biopsies of embryos for sexing, thus avoiding the problem of trying to digest an intact zona pellucida. The other major difficulty in trying to karyotype embryos was due to a lack of metaphases. Hare et al. (1980) noted that the ability to sex embryos is related to the number of cells available for sexing. Metaphase spreads appeared to be in better condition before staining under phase contrast than after Giemsa staining. It may be that too long an exposure to stain (1h) and change in pH of stain after long storage affected embryonic chromosomal morphology.

Even in experienced hands, only about 60% of embryos can be sexed and can be transferred fresh with about 60% pregnancy rate (Picard et al., 1984; Rall and Leibo, 1987). This causes a problem for dealing with the 40% unsexable embryos. If 60/100 embryos are sexable, and only the 30 of the desired sex are transferred then 30 recipients will be required, and only 18 calves of the desired sex are born from 100 embryos. If the 30 embryos of the desired sex and the 40 unsexed embryos are transferred, then 70 recipients will be required and 30 (18 from sexed embryos and 12 from unsexed embryos) calves of the desired sex and 12 of the undesired sex will result. If embryos were not sexed then at a 60% pregnancy rate 100 recipients will be
required and 30 of the desired sex and 30 of the undesired sex of calf will be born. Usefulness of the karyotyping procedure will depend on the efficiency and cost of the sexing procedure, the value of the desired and undesired sexes, and the cost to maintain recipients. The karyotyping technique would increase in usefulness if procedures can be developed which increase sexability rate.
Chapter 4

CONCLUDING DISCUSSION

Initiation of superovulatory treatment in the presence of a dominant follicle has been shown to decrease superovulatory responses (Grasso et al., 1989; Guilbault et al., 1991; Huhtinen et al., 1992) although other authors have reported no effect of a dominant follicle on superovulatory responses (Wilson et al., 1990). Two experiments were carried out to investigate the success of superovulation in the absence of a dominant follicle.

Initiating superovulation treatment in the early estrous cycle to avoid the influence of a dominant follicle

In the first experiment, follicular growth, ovulation and embryo recovery rates were compared between cows which were superovulated at Day 2 of the estrous cycle, before a dominant follicle is present, and control cows superovulated at mid-cycle when a dominant follicle may have been present. Previous researchers have noted that there were poorer superovulatory responses when superovulation was initiated at the beginning of the estrous cycle (Philippo and Rowson, 1975; Sreenan and Gosling, 1977; Lindsell et al., 1985). Recently, Goulding et al. (1990) reported that fewer corpora lutea and embryos were found after slaughter in cows superovulated at Day 2 of the cycle compared to Day 10. However, follicular development during superovulation treatment was not measured in any of these studies.

In this experiment, follicular development and ovulation
rate were monitored after superovulation induction in cows beginning superovulatory treatment at Day 2 or at mid-cycle Days 9-13. All cows responded to FSH with adequate follicular development. However, few cows which were superovulated on Day 2 demonstrated estrus and most failed to ovulate. Regressing follicles were noted at the expected time of embryo collection. Very few embryos were recovered from cows which were superovulated early in the cycle. This experiment demonstrated that although follicular development was adequate in response to superovulation induction early in the cycle, ovulation rates were low. Lack of ovulation of the FSH-stimulated follicles after superovulation on Day 2, may have been due to several factors. Most of the Day 2 cows failed to demonstrate estrus. Using ultrasound, it was evident that in many cows, the corpus luteum failed to undergo complete luteolysis. Lindsell et al. (1985) noted that few animals superovulated on Day 3 of the estrous cycle demonstrated estrus. Prostaglandin F$_2\alpha$ was given on Day 7 of the estrous cycle in Day 2 cows, at a time when the CL is normally responsive (King et al., 1982; Momont et al., 1984). Therefore, it is likely that administration of gonadotrophins early in the estrous cycle may have affected CL response to PGF$_2\alpha$. Also it was likely that many of the follicles stimulated by Day 2 FSH treatment were atretic because they were unable to ovulate following hCG.

Removal of the dominant follicle at Day 7 of the estrous cycle with hCG prior to superovulatory treatment

In the second experiment, superovulation parameters were
compared between animals which began treatment at mid-cycle, when a dominant follicle may have been present; and animals in which the dominant follicle was removed with hCG on Day 7 of the estrous cycle before initiation of superovulation. Human chorionic gonadotrophin previously has been used to ovulate the dominant follicle present on Day 7 of the cycle and increase progesterone levels (Rajamahendran and Sianangama, 1992). Progesterone values during superovulation treatment have previously been shown to affect superovulatory responses by some workers (Yadav et al., 1986; Goto et al., 1987) but not by others (Sreenan and Gosling, 1977; Saumande et al., 1985; Tamboura et al., 1985). In this experiment, progesterone values during superovulation treatment did not significantly affect superovulation responses.

Cows which had the dominant follicle removed by hCG treatment prior to superovulation induction tended to have better follicular development, ovulation rate and embryos recovered, however these values were not significantly different between control cows and hCG-treated cows. Better superovulatory responses have been noted at Days 8-12 (Philippo and Rowson, 1975; Lindsell et al., 1985) than at other days of the cycle. Goulding et al. (1990) have postulated that this response "window" is related to the time in the estrous cycle when a dominant follicle may not be present. Timing of the "window" may vary depending on whether the estrous cycle will have two or three waves of follicular development. Optimal responses may have occurred in both the hCG-treated and control
groups in this experiment because all cows were superovulated beginning on Days 8-11. In addition, the beneficial effect of initiating superovulation in the absence of a dominant follicle may have been confounded by the effect of hCG on small antral follicles. Investigation of the effect of initiating superovulation in the absence of a dominant follicle might better be resolved by removal of the dominant follicle through other means such as laparotomy and electrocautery or through ultrasound-guided follicular aspiration.

It would be useful if a method to allow superovulation in the absence of a dominant follicle could be selected without reference to ultrasound monitoring. Because a dominant follicle is usually found on Day 7 of the estrous cycle in either two or three wave cycles, it may be possible to use an agent such as hCG to remove the dominant follicle to create a more appropriate environment for initiation of superovulation.

Conclusions from superovulation experiments

Both superovulation experiments were conducted at a time in the estrous cycle when a dominant follicle would not be present. Although administration of gonadotrophins early in the estrous cycle caused follicular development, the majority of these FSH-stimulated follicles were unable to ovulate. However, in the second experiment, removal of the Day 7 dominant follicle with hCG did not significantly increase the numbers of follicles, corpora lutea or embryos recovered from superovulated donors. It may be that the dominant follicle does not have a large
effect on superovulatory success, hCG itself affects superovulatory success, or other intrinsic factors affect superovulatory parameters. Investigations on other methods of removing the dominant follicle may help resolve this issue.

Sexing preimplantation embryos

H-Y antisera raised in mice have been used previously in several experiments to sex preimplantation embryos (White et al., 1983, 1987) with 80% accuracy. Attempts were made to immunize C57BL/6 female mice against male splenic cells to raise H-Y antisera. The titre of the antisera was then measured in two ELISA systems. Both ELISAs demonstrated that there was high non-specific cross-reactivity in sera from control and immunized mice. H-Y immunized females were bred to assess the effect of maternal H-Y antibodies in vivo on litter size and sex ratio. The litter size was unaffected by treatment. There were increased numbers of males in the first litter of immunized females. This may have been due to beneficial immunological reaction of the mother to H-Y antigen on male fetuses. Tolerance may have been induced to H-Y antigen due to repeated exposures of immunized females, and this may be the reason for normal sex ratios in later litters.

Another method used to sex embryos before embryo transfer is karyotype analysis. A few cells can be removed from bovine preimplantation embryos to perform karyotype analysis. In cattle, the X- and Y-chromosome are easily identified in good chromosomal preparations. Previously, using karyotyping
techniques to assess embryonic sex, about 60% embryos could be successfully sexed (Picard et al., 1984; Rall and Leibo, 1987). In this experiment, whole embryos were used for karyotype analysis. There were some difficulties in preparing good chromosomal spreads. Sexing embryos by karyotype analysis is limited by the small number of cells available. About 30% of processed embryos had chromosomal spreads but chromosomes were indistinct after staining and few embryos were easily sexable.
References


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