AN ABSTRACT OF THE THESIS OF

Maria K. Gomes for the degree of Master of Science in Animal Science presented on March 16, 2016.

Title: Modifying the Hormone Strategy for Superovulating Donor Cows to Reduce Drug Costs without Decreasing the Number of High Quality Transferable Embryos Recovered

Approved:_____

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Two research projects comprise this thesis. The first project investigated modifying the hormone dosing strategy traditionally used in superovulating donor cows for an embryo collection to decrease drug costs without decreasing the number of high quality, transferable embryos recovered. The objective of this project was to evaluate the number and quality of embryos recovered from donor cows superovulated with a reduced dose of the pituitary gonadotropic hormone, follicle stimulating hormone (FSH), and a greater dose of the hypothalamic releasing hormone, gonadotropin releasing hormone (GnRH). In Experiment 1, 24 crossbred beef cows from the Oregon State University Beef Cattle Ranch were assigned to one of four superovulation treatment groups according to age and parity: 1) 400 mg FSH and 100 µg GnRH (the traditional doses), 2) 400 mg FSH and 200 µg GnRH, 3) 200 mg FSH and 100 µg GnRH, or 4) 200 mg FSH and 200 µg GnRH. Embryos were collected non-surgically 7 d after estrus onset and scored for stage

of development and quality using a four-rank grading scheme. In Experiment 2, 12 crossbred beef cows from the Oregon State University Beef Cattle Ranch were superovulated twice with either 1) 400 mg FSH and 100 µg GnRH or 2) 200 mg FSH and 100 µg GnRH. Embryos were collected non-surgically as described in Experiment 1 and good to excellent quality late morulae to blastocysts were cultured, one embryo per 15-microliter drop, for 8 d. At 24-h intervals, embryos were evaluated for viability and overall development and transfered to a fresh micro-drop and conditioned medium was recovered. Conditioned media were assayed for plasminogen activator (PA), a protease correlated with embryo cell number and development to advanced cell stages. In Experiment 1, cows superovulated with 200 µg GnRH produced more unfertilized ova (P = 0.02) than cows superovulated with 100 µg GnRH. Cows superovulated with 200 mg FSH produced a higher percentage (P = 0.07) of transferable embryos and a lower percentage (P = 0.10) of degenerate embryos than cows superovulated with 400 mg FSH. Superovulating cows with the reduced FSH and GnRH doses (200 mg and 100 µg, respectively) yielded fewer total transferable embryos (1.8 embryos) but a greater percentage of transferable embryos at a reduced cost compared to the traditional dose ($24 vs $31 per embryo, respectively). Increased GnRH dosing had a negative effect on transferable embryos and the reduced FSH and increased GnRH dosing was not cost effective. In Experiment 2, cows superovulated with 400 mg FSH produced more unfertilized ova (P = 0.08) than cows superovulated with 200 mg FSH. More (P=0.04) embryos recovered from cows treated with 200 compared to 400 mg FSH developed to the hatched blastocyst stage in culture. Embryos recovered from cows treated with 200 compared to 400 mg FSH also developed to the expanded blastocyst stage sooner (P =

0.08). Embryos collected from cows receiving 200 mg FSH produced more (P = 0.04) PA in the first round of superovulation compared to 400 mg FSH and both doses in the second round. Similar to Experiment 1, the 200 mg FSH dose yielded fewer total transferable embryos in the first round of superovulation but at a reduced cost compared to the 400 mg dose ($25 vs $37 per embryo, respectively). Collectively, these data suggest higher FSH dosing is likely inducing ovulation of poorer quality ova which either fail to fertilize or, if fertilization occurs, may generate a reduced percentage of competent embryos. The reduced FSH dose not only contributes to a reduced cost but may also provide more embryos with a greater likelihood of pregnancy establishment.

The second project in this thesis attempted to develop a dipstick-style enzyme assay to assess embryo viability prior to transfer. The objective of this project was to develop a dipstick that rapidly quantified PA production by an embryo and could be used on the farm for an embryo collection and transfer. Dipsticks were constructed by cutting and mounting 5 X 5 mm squares of cellulose acetate, chromatography paper, glass fiber membrane or nitrocellulose on the end of 5 X 25 mm plastic strips. Five microliters of 1, 10 or 50mM of the tripeptide glutamic acid-glycine-arginine (EGR; $C_{27}H_{36}N_8O_7 \bullet CH_3COOH$), a colorimetric substrate for urokinase (UK), were pipetted onto the 5 X 5 mm squares and dried. Dipsticks were incubated in 25-μL of culture medium containing 0, 1, 10, or 100 IU UK/mL or embryo-conditioned medium (ECM) and visually observed for color development at 30-min intervals for up to 90 min. Color development was scored using the following 3-point system where: 0 = absence of yellow, 1 = light yellow and 2 = bright yellow. Dipsticks were able to detect EGR cleavage in 10 and 100 IU/mL UK after 90 and 30 min of incubation, respectively, but no

color was observed in dipsticks incubated in ECM. A second approach was developed using a 96-well plate. Twenty-five microliters of 1, 5, 10, 20 or 50 mM EGR were combined with 25-μL of culture mediumcontaining 0, 1, 10, or 100 IU UK/mL or ECM and observed for color development visually and photometrically at OD 405 using an ELISA plate reader at 30-min intervals for up to 120 min. Color development was visually observed in 1, 5, 10 and 50 mM EGR after 30 min of incubation with 100 mM UK/mL but no color was observed in wells containing ECM. However, when the plates were evaluated photometrically EGR cleavage was detected in wells containing ECM and 50 mM EGR after 30 min of incubation. Although the PA dipstick enabled visual detection of EGR cleavage by 10 and 100 IU/mL UK, it was not sensitive enough to detect PA in ECM. The 96-well plate assay was successful in detecting PA in ECM after 30 min of incubation but only photometrically. To use the plate-based assay would require the embryo transfer practitioner to have an ELISA plate reader on hand and the expense associated with such an instrument may limit its on farm application.

MODIFYING THE HORMONE STRATEGY FOR SUPEROVULATING DONOR COWS TO REDUCE DRUG COSTS WITHOUT DECREASING THE NUMBER OF HIGH QUALITY TRANSFERABLE EMBRYOS RECOVERED

By

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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Maria Gomes, Author

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CHAPTER 1

INTRODUCTION

This thesis is composed of two independent projects. The first project examined modifying the dosing strategy to superovulate donor cows without decreasing the number of high quality transferable embryos recovered, and the second project was aimed at designing an assay to quantify urokinase-type plasminogen activator (uPA) in cultured embryo medium. Initial attempts in the latter project were dedicated to using a double antibody assay, however, it was discarded in favor of an enzymatic assay.

Superovulation, prior to artificial insemination, is a routine procedure in embryo transfer, in order to obtain more than one calf from genetically superior cows each year (Bó et al., 2013). This technology enables herd improvement in that it allows an individual to quickly obtain a herd of genetically superior cows, improve female reproductive efficiency, and obtain more offspring from elite sire-dam matings, thereby reducing the number of unwanted calves in a beef or seedstock operation. Alternatively, extra embryos produced by superovulated cows can be cryopreserved and transfered to recipients in the future.

Arguably the most critical part of an embryo transfer procedure is the first part, or embryo collection, when cattle embryos are collected from superovulated donors. In order to enable cows to ovulate more than a single ovum every estrous cycle, donors commonly undergo a four-day follicle stimulating hormone (FSH) regimen. Practitioners will usually administer a second hormone, gonadotropin releasing hormone (GnRH), when the donor cows come into heat, in order to increase the number of ova ovulated and

increase the total number of embryos produced and collected from a donor during a single superovulation protocol.

However, this technology does not come without its drawbacks. Despite the popularity of injecting GnRH, a clear advantage of this injection on improving overall embryo production has not been observed. This lack of clear response could be due to the variation associated with superovulatory responses in individual cows making it difficult to observe differences, or it could be due to insufficient dosing of GnRH. In our efforts to increase the ovulation rate of cattle, we may not be giving enough GnRH. Another issue with the current superovulation protocol is that the lowest dose of FSH needed to superovulate a cow is not currently known (Barati et al., 2006). A third setback is the high price of Folltropin V®, the pharmaceutical FSH preparationused in superovulation, making the current superovulation protocol expensive to carry out, especially in a large commercial setting (Armstrong, D. T., 1993). In contrast, GnRH is inexpensive to purchase in the form of Fertagyl®, Cystorellin®, or Factrel®. The cost of Folltropin V® needed to superovulate one cow is currently $172, while the cost of Fertagyl® is about $3.50/cow. The objective of the first project was to evaluate the number and quality of embryos recovered from donor cows superovulated with reduced dosing of FSH and increased dosing of GnRH. The hypothesis of Experiment 1was the altered superovulation protocol, using less FSH and more GnRH than the standard protocol, will yield fewer total ova, but more high quality, transferable embryos while costing less. The hypothesis of Experiment 2 was cows superovulated with the higher FSH dose would yield poorer quality embryos as evidenced by measures of in vitro vitality.

An important aspect of embryo transfer is examining and grading embryos prior to transfer or freezing. Currently, embryos are graded according to a scheme developed by Lindner and Wright (1983), which categorizes embryos as poor, fair, good, or excellent or by a scheme developed by Shea (1981) which assigns ranks of 1, 2, 3, or 4 to each embryo. However, neither of these grading schemes is highly accurate; according to Renard et al. (1980) these methods of assessment are subjective and dependent on having previous experience. For example, 45% of transfered embryos graded as "excellent" generated a pregnancy and 71% of embryos graded as "4" (perfectly symmetrical, evenly granulated, lacking in blastomere extrusion and possessing no deformation in the zona pellucida) generated pregnancies (Mendoza et al., 2012).

Actively developing embryos produce more of the extracellular serine protease, plasminogen activator (PA) than poorly developing embryos (Kaaekuahiwi and Menino, 1990). The amount of uPA produced by an embryo is positively correlated with embryonic size, developmental stage and cell number. To that end, the second project was devoted to constructing a dipstick style assay to quantify the amount of PA present in medium.An alternative assay was also developed using a 96-well plate. For both assays, the synthetic colorometric PA substrate, glutamic acid-glycine-arginine (EGR) was used because cleavage by PA produces a yellow color. The goal of this project was to develop a simple, rapid, assay for measuring embryo viability, to be used on the farm during an embryo transfer, which would supersede current grading practice.

CHAPTER 2

LITERATURE REVIEW

The Estrous Cycle of the Cow

The cow's estrous cycle has an average duration of 21 days and is divided into two phases: follicular and luteal (Senger, 2003). Ovarian follicles develop throughout the estrous cycle in two or three waves consisting of at least one dominant follicle and several subordinate ones (Adams et al., 1992, Boer et al., 2011). Each wave of follicular development is preceded by an increase in FSH, which is released from the anterior pituitary gland. The follicle which ovulates is the dominant follicle of the second or third wave for females with two or three waves, respectively, in their estrous cycles. Moreover, the preovulatory FSH surge is coincident with the preovulatory surge of luteinizing hormone (LH), which is also released from the anterior pituitary gland. Secretion of estradiol (E_2) from growing follicles characterizes the follicular phase, whereas the luteal phase is characterized by progesterone (P_4) secretion from the corpus luteum (CL) in the ovary which prepares the uterus for pregnancy (Senger, 2003). Cattle are classified as nonseasonally polyestrous, which means they are not influenced by photoperiod and females have estrous cycles which repeat every three weeks, on average, allowing them to become pregnant at any time during the year.

In addition to being broken into the follicular and luteal phases, the estrous cycle of the cow can also be divided into proestrus, estrus, metestrus, and diestrus. Estrus is the period when the female allows copulation, and lasts about 12-16 h (Senger, 2003). $E_2$is the dominant hormone during estrus, and estrus is the most recognizable stage of the estrous cycle because it is characterized by visible behaviors, like sexual receptivity to

the male and mating. At the onset of estrus, a cow will display behaviors indicative of her impending sexual receptivity, such as walking around more, vocalizing more, and attempting to mount other cows. During estrus, the cow will be more willing to mate, a time referred to as standing estrus. Unlike most of the females, ovulation in cattle occurs during the following phase, metestrus. Metestrus is also the time of transition from E_2 dominance to P_4 dominance, and is when a functional CL forms through the process called luteinization. P_4 secretion can be detected soon after ovulation, but 2-5 days are usually required after ovulation for the CL to produce significant quantities of P_4. Diestrus is best described as the period of maximal luteal function, and is characterized by a high plasma concentration of P_4. Diestrus lasts 10-14 days and the high concentrations of P_4 cause the uterus to prepare itself for early embryo development. Diestrus ends when the CL is broken down via luteolysis. Luteolysis is the breakdown of the CL caused by prostaglandin $F_{2\alpha}$($PGF_{2\alpha}$), and this results in the loss of the structure and function of the CL. Proestrus is the period of time right before estrus, and is brought about by declining progesterone levels resulting from the CL undergoing luteolysis (Senger, 2003). Proestrus lasts 2-5 days and is characterized by switching from a period of P_4 dominance to a period of E_2 dominance. It is during proestrus that follicles are recruited for ovulation.

During the follicular phase of a heifer's first estrous cycle, a wave of follicles will start to grow in the ovaries under the influence of FSH (Boer et al., 2011). Luteinizing hormone is also released in bursts called pulses. As they grow, follicles produce E_2 and inhibin, and both hormones are released into the heifer's peripheral bloodstream. Follicle stimulating hormone stimulates all the follicles in a certain size range to grow, while inhibin inhibits FSH, so that less FSH is present in the peripheral bloodstream to induce

follicular growth. Inhibin secreted by dominant follicles suppresses subordinate follicles through systemic endocrine channels by selectively suppressing the synthesis and release of FSH, causing subordinate follicles to cease growing and begin degenerating (Adams et al., 1992). The follicles produce more FSH receptors as they grow, allowing them to continue growing despite decreasing amounts of FSH (Boer et al., 2011). As the follicles grow bigger they also produce E_2, which triggers the release of gonadotropin releasing hormone (GnRH) from the hypothalamus, inducing more LH secretion from the pituitary gland. After a few days, the follicle with the most FSH receptors becomes the dominant follicle, and is ovulated following a surge of LH, since there is no P_4 present at this time. Follicles have a diameter of less than 1mm when they are first stimulated to grow by FSH, but dominant follicles can have diameters of 10mm or more by the time they are ovulated (Nivet et al., 2012). After ovulation, the ovarian theca interna and granulosa cells previously surrounding the oocyte undergo hyperplasia and hypertrophy to become small and large luteal cells, respectively (Stötzel et al., 2012), to collectively form a corpus hemorrhagicum (CH) (Senger, 2003). The CH begins producing P_4, which inhibits dominant follicles in subsequent waves from ovulating (Senger, 2003; Boer et al., 2011). In 2-3 days the CH will transform into the corpus luteum (CL), which produces more P_4 and induces atresia in any follicles developing while the CL is functional (Boer et al., 2011).

Although waves of follicle development are constantly occurring, the presence of the CL on the ovary both lends its name to and indicates the beginning of the next phase of the bovine estrous cycle: the luteal phase. During the luteal phase, the ovulated oocyte travels down the ampullary portion of the oviduct, eventually reaching the ampullary-

isthmic junction, the common site for fertilization in the cow (Senger, 2003). If the oocyte remains unfertilized, the absence of an embryonic signal allows oxytocin from the posterior pituitary gland to induce prostaglandin$F_2\alpha$ ($PGF_2\alpha$) secretion from the uterus (Boer et al., 2003). $PGF_2\alpha$ is released in a pulsatile manner and causes the CL to regress, effectively terminating P_4 secretion on day 17 or 18 of the estrous cycle (Stötzel et al., 2012).

The absence of P_4 will allow the dominant follicle from the next wave of follicular development to ovulate its oocyte, and the cycle will start over as the next follicular phase ensues (Senger, 2003). Because no pregnancy has occurred, the regressing CL transforms into a white, scar-like structure called a corpus albicans (CA) (Senger, 2003). However, in order for $PGF_2\alpha$ to effectively regress the CL, the CL must have been exposed to P_4 for ten to thirteen days (Boer et al., 2003). P_4 has a negative effect on the GnRH neurons in the hypothalamus, suppressing the production of LH and FSH, thereby allowing the production of little E_2 (Senger, 2003). As a result of the action of $PGF_2\alpha$, plasma P_4 levels fall sharply (Stötzel et al., 2012), while plasma E_2 levels rise (Boer et al., 2003).

If the oocyte is fertilized the CL will be free from the inhibiting effects of $PGF_2\alpha$ and will continue secreting P_4 for about ten days post-ovulation, just the same as it does without fertilization (Senger, 2003). The embryo will reach the cow's uterus three to four days after it was ovulated as an oocyte (four to five days after the cow was in heat) (Seidel, 1981). Interestingly, it can take up to seven days for all the embryos in a superovulated cow to reach the uterus (Betteridge and Flechon, 1988). Sometime between days 13 to 21 after ovulation, the trophoblast cells of the bovine embryo secrete

Interferon-Tau, a pregnancy recognition protein that prevents uterine $PGF_2\alpha$ secretion (Senger, 2003). Interferon-tau production acts as an antiluteolytic agent in a cascade fashion by suppressing E_2 receptors so uterine oxytocin receptors are not synthesized, thereby preventing oxytocin from stimulating $PGF_2\alpha$ synthesis.

The time between ovulations is called the interovulatory interval, and cows will experience two or three waves of follicular development during this time (Adams et al., 1992). Waves of follicular growth begin on days 0 and 10 in animals with two wave cycles and days 0, 9, and 16 in animals with three wave cycles (Adams et al., 1992). Three or four days after follicular growth has started in the luteal phase, the subordinate follicles stop growing and the dominant follicle undergoes atresia (Adams et al., 1992). There is always a medium or large-sized follicle present in the ovary (Boer et al., 2003).

History of Bovine Superovulation

Embryo transfer (ET) refers to the collection of embryos from a donor female followed by the transfer of embryos to recipient females for development to term. A valuable donor is superovulated, or induced to ovulate more than one ovum per estrous cycle, and is then bred at estrus with semen from a genetically valuable bull. The embryos are collected after 6-8 days of development, assessed for quality, and may be transfered directly to a recipient whose estrous cycle was synchronized with the donor, or they can be frozen in liquid nitrogen for transfer later. The female who underwent a superovulation procedure can undergo a normal breeding at her next estrus or can be superovulated again in two or three months' time.

Due to the relatively long generation interval possessed by cattle and their low reproductive rate making it difficult to quickly improve a herd or increase the size of a

herd in response to market demand, many researchers and producers felt it necessary to increase the reproductive efficiency of the world's beef and dairy herds through embryo transfer (Seidel, 1981). For this reason, a preliminary superovulatory protocol was developed. The first superovulation protocol has undergone many changes since its inception, and superovulation is no longer a novel practice in bovine embryo transfer (Seidel, 1981). The 80% increase in bovine pregnancies in North America between 1978 and 1979 may be mostly attributed to the improvement and implementation of superovulation and embryo transfer (Seidel, 1981). Unfortunately, in spite of the steady increase in occurrence of superovulation protocols in the past forty years, the average number of transferable embryos recovered from a single cow has been stagnant at about six per flush, with a range of 0-60 embryos recovered per cow (Hesser et al., 2011). At the time of the advent of these procedures, when it was first becoming popular to use superovulation and embryo transfer to produce animals for sale at markets, a "boom and bust" cycle left many entrepreneurial cattle producers poorer than when they started. This was because the market was now saturated with bull and heifer calves possessing traits which had formerly been hard to attain (Seidel, 1981). When superovulating cows, and other monotocous species, one can now expect to see ovulation rates up to 5-10 times greater than normal (Senger et al., 2003).

The first successful embryo transfer procedure was performed at Cambridge University in rabbits 123 years ago, in 1891, by Sir Walter Heape; however, it wasn't until 1951 when the first successful bovine embryo transfer was performed (Seidel, 1981).The earliest reports of superovulation protocols for cattle came from Wisconsin (Bó et al., 2014). Although much has changed since then, the goal of superovulation

remains the same, that is, to obtain as many transferable embryos with high probabilities of generating pregnancies as possible. Unfortunately, the success rate of superovulation is quite variable (Desaulniers et al., 1995), as some cows may ovulate more than ten oocytes and others may not ovulate at all. Moreover, a cow which ovulated a high number of oocytes after being superovulated one time might not ovulate any the next time, since the results of using gonadotropins such as FSH and equine chorionic gonadotropin (eCG) are inconsistent (Sendag et al., 2008). Multiple groups of researchers have cited the variability of superovulatory response as the most frustrating part of their research projects, and another group of researchers pointed out that the success rate of using superovulation to generate transferable embryos is still relatively low, ranging from 20-40% (Greer et al., 1992). The first bovine protocol recorded involved the administration of gonadotropins several days before the cow was expected to come into estrus (to induce more than one ovulation) and an injection of human chorionic gonadotropin (hCG) on the day of estrus onset, which was expected to induce ovulation (Bó and Mapletoft, 2014).

Adams and colleagues (1992) researched the relation of dominant follicle ablation to a subsequent surge of FSH. Adams et al. (1992) found that a surge of FSH occurred in a group of cattle which had undergone dominant follicle ablation 2-4 days before the FSH surge occurred in cattle which had not undergone dominant follicle ablation.

Despite the variability in ovarian response, Goodhand et al. (1998) reported that Simmental heifers treated with FSH for three consecutive days before undergoing a weekly follicular aspiration procedure produced more follicles than Simmental heifers undergoing weekly follicular aspirations without FSH, and more oocytes from the FSH-

treated heifers were graded as Category 1, meaning they had at least four cell layers of good cumulus and a clear cytoplasm. In addition, heifers treated with FSH produced more transferable morulae and blastocysts than heifers not treated with FSH.

The first widely used superovulation protocol was developed by Hafez and his colleagues in the 1960's, and involved the administration of 2000-3000 IU of eCG on Day 16, followed by 10mg of E_2-17-β on days 19 and 20 of the estrous cycle (Bó and Mapletoft, 2014). On day 21, 2000 IU of hCG was administered. It was during these procedures that researchers noticed the estrus-inducing effects of E_2-17-β on their subjects; it was initially given to ensure luteal regression.

Elsden and colleagues (1978) found that cows treated with $PGF_2\alpha$ two days after receiving eCG exhibited higher superovulation rates than cows which received eCG alone (Bó et al., 2014), causing a $PGF_2\alpha$ injection to be added to the current superovulation protocol. It was discovered that the time of $PGF_2\alpha$ administration corresponded approximately to the time of emergence of the second follicular wave. Elsden and others (1978) also discovered that an equine pituitary extract consisting of 80% FSH and 20% LH was more successful in superovulating cattle than eCG, and these results were quickly found to be true by other researchers. The rapid exchange of FSH for eCG in the commonly used superovulation protocol was also due to the shorter half-life of FSH; FSH has a half-life of 3-4 h, whereas the half-life of eCG is 40 h. Short half lives are important because once a hormone is released into the body system and causes a response, it needs to be quickly degraded to prevent further, unnecessary responses (Senger et al., 2003). Additionally, if a receptor is exposed to a hormone for a prolonged period of time, it could become desensitized, no longer allowing the hormone to exert the

desired effect. Sendag et. al (2008) compared the superovulatory responses of ten German Holstein cows superovulated with FSH to that of 14 German Holstein cows superovulated with eCG and found cows superovulated with FSH had a greater superovulatory response, as measured by the production of more oocytes. They also found that cows treated with FSH had more ovarian follicles and oocytes of a superior quality than those from cows treated with GnRH.

By 1996, it was common knowledge in the scientific community that superovulated cows treated with gonadotropins could be a source of large numbers of oocytes with a high level of developmental competence (Bordignon et al., 1996). As many as 50 calves in one year have been obtained from one cow, while multiple cows have produced ten calves in one year (Seidel, 1981). Rather than focusing on the genetic potential an animal resulting from a superovulation could possess, most donor cows are chosen on the basis of how much money their offspring might bring to their owner (Seidel, 1981).

Common Uses For Superovulation

Another reason to use superovulation, besides obtaining more high quality embryos from animals with superior genetics, is to produce embryos from donors who would otherwise be infertile (Seidel, 1981). It would be very expensive and foolish to propagate the genetics of cows which are infertile due to a genetic problem, but if a valuable cow becomes injured, develops a pathology, or reaches ovarian senescence, superovulation can be used to produce viable embryos from that cow. In these cases, an animal produces viable ova but may be unable to carry a pregnancy. It is important to note that not all embryos produced by superovulating a senescent cow will be viable, but

it is possible to produce a pregnancy when morphologically normal embryos collected from senescent donors are transfered to viable recipients. While the results of superovulation and embryo transfer procedures using senescent donors are not spectacular, one individual reported obtaining 30 pregnancies over the course of 15 months from one infertile donor (Seidel, 1981).

Superovulation and embryo transfer have also led to shipping frozen or non-frozen embryos around the world, as it is much cheaper to ship an embryo than it is to ship a full-grown cow (Seidel, 1981). The number of deaths of non-indigenous animals is reduced when embryos are shipped instead of adult animals; an adult animal suddenly introduced into a new environment with different local pathogens and illnesses will not fare as well as an embryo transfered into an indigenous adult animal which obtains the benefit of the indigenous animal's colostrum, adapting its immune system from birth to be able to cope with local diseases and pathogens. While artificial insemination is still the method of choice for introducing new genetics into a herd, results will be seen much sooner with embryo transfer. It takes three generations, using artificial insemination, to obtain a calf that has seven-eighths of the genetic makeup of a new breed, whereas embryo transfer yields a calf that is genetically one hundred percent of the new breed, and will be able to introduce its genetics much more rapidly in the existing herd.

One final common application for superovulation is testing cows and bulls for Mendelian recessive alleles (Seidel, 1981). If a cow is thought to be heterozygous at a certain locus for an undesirable trait, she must produce eight normal offspring when bred to a bull known to be homozygous recessive for the same trait. Without superovulation, many cows would not be able to produce that number of offspring in their productive

lifetime, and if they did manage to, there would be eight animals who would definitely be carriers, and therefore undesirable to breed. Instead, it is reasonable to expect eight embryos to be produced in a year or two from a suspected carrier when superovulated. If all eight are negative that animal will still have many good, productive years left in which to give offspring which can eventually be bred without the fear of a recessive allele expressed in their offspring.

Bulls thought to be carriers of Mendelian recessive alleles also must produce eight phenotypically normal offspring when bred with a homozygous recessive cow before being accepted as homozygous dominant at that particular locus. Cows which are homozygous recessive at any locus are much harder to come across than homozygous recessive bulls, so it is more difficult to perform this kind of test on a bull. However, homozygous recessive cows may be superovulated and bred with semen from bulls whose genetic makeup is in question, allowing the genetic makeup of these bulls to be ascertained much sooner. Furthermore, many traits being analyzed for, such as dwarfism and mule-foot, can be observed in a sixty-day-old fetus, so the recipient of such an embryo can be slaughtered and the effects on the fetus observed without harming the rare donor (Seidel, 1981).

A Common Synchronization and Superovulation Protocol

Several superovulation protocols commonly used today involve the use of the following three hormones or analogues of these hormones (Bo et al., 2014): $PGF_2\alpha$ (commonly given in the form of Lutalyse® or Estrumate®), FSH (commonly given in the form of purified porcine FSH and marketed as Folltropin V®), and GnRH (commonly given in the form of Fertagyl®, Factrel® or Cystorellin®). GnRH is a decapeptide

produced by the hypothalamus which induces the anterior pituitary to secrete FSH and LH (Dias et al., 2010). FSH is a glycoprotein polypeptide hormone that is synthesized and secreted by the gonadotropic cells of the anterior pituitary gland, and LH is also a hormone produced by the gonadotropic cells in the anterior pituitary gland.

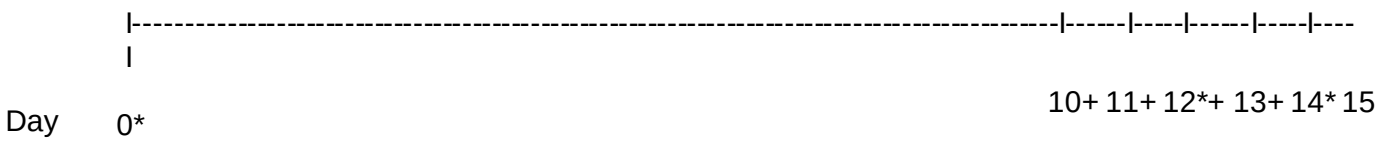

Figure 1. Twelve-day double Lutalyse injection protocol. An asterisk (*) denotes a Lutalyse® injection, a plus sign (+) denotes two Folltropin V® injections, and each vertical line (I) represents a day.

The protocol (Figure 1) begins with an injection of 25mg Lutalyse® given to each cow to be superovulated (Bo et al., 2014), and the day of this first injection is designated as Day 0. Recipients (cows scheduled to receive embryos) also receive a 25mg injection of Lutalyse® on the same day as the donor animals (Senger et al., 2005). The purpose of this Lutalyse® injection is to regress any existing CL and cease P_4 production, making it possible to initiate superovulation treatment at any time in the estrous cycle (Bo et al., 2014), as well as to synchronize recipient and donor animals. The $PGF_2\alpha$ injection at the beginning of the protocol was an extremely important milestone because before the availability of $PGF_2\alpha$ superovulation protocols were initiated four or five days before the cow was expected to come into estrus, a time that could be difficult to predict (Seidel, 1981), or P_4 protocols were used. However, the implementation of a $PGF_2\alpha$ injection has provided flexibility on the part of the donor animals and allowed the superovulatory

protocol to improve in efficacy because the protocol no longer had to be initiated at a certain day in the cow's estrous cycle. On the morning of Day 10, each donor is given 50mg Folltropin V® intramuscularly (Bo et al., 2014), and 12 h later each cow is given another intramuscular injection containing 50mg Folltropin V®. This twice-daily injection series is repeated for three more days, so that over the course of Days 10-13, each cow to be superovulated receives a total of 400mg Folltropin V®. Additionally, a second Lutalyse® injection is given to each cow, in the morning or evening of Day 12, along with the Folltropin V® injection, and a third Lutalyse® dose is given on the morning of Day 13 as well. The FSH injections are administered because they are required for follicle recruitment and growth until the dominant follicle reaches 8.5 mm in diameter in Bos taurus cattle or a diameter of 6.2 mm in Bos indicus cattle. The injections allow more than one follicle to grow to preovulatory size.

Twenty-four hours after the second Lutalyse® injection has been given, cows undergoing the protocol are monitored every 12 h for signs of standing estrus, or heat (Hesser et al., 2011). The most common sign of estrus is when a cow stands to be mounted by other cows, but other signs include raised tailhead, swollen vulva, or mucus dangling from the vulva (a "bull string"), as well as walking around more and being restless. Donors commonly come into estrus 36-48 h after the second Lutalyse injection, and, once cows are observed in heat, they are typically artificially inseminated with semen from the same bull at 0, 12, and 24 h after estrus detection (Seidel, 1981). However, cows can be naturally serviced at the appropriate time following superovulation, and it is not unheard of to artificially inseminate cows with a mixture of semen from two or three bulls and record the sire of the calves after they are born via

blood typing. At the time of the first breeding, each cow is also given an intramuscular injection containing 100 µg GnRH (Funston et al., 1995). The purpose of the GnRH is to augment endogenous FSH and LH (Senger, 2002), which would induce follicles close to ovulatory size to rupture and release an oocyte (Boer et al., 2003), thereby increasing the number of potential embryos (Bordignon et al., 1996). Additionally, GnRH injections have been shown to positively impact the developmental competence of oocytes.

Currently, it is common to collect the prospective embryos via a non-surgical uterine flush seven days after the first artificial insemination is performed on an animal (Hesser et al., 2011, Cushman et al., 2000). Before collection, each animal is rectally palpated to determine the number of corpora lutea present on each ovary (Lubbadeh et al., 2014). This number is recorded and serves as an estimate of the number of embryos expected to be recovered from the uterine flush (Lubbadeh et al., 2014). Typically, in the OSU reproduction lab, 1000 mL of Dulbecco's phosphate buffered physiological saline (DPBS) containing heat treated fetal calf serum, antibiotics and antimycotics are introduced into the cow's uterus via a sterile plastic catheter, in increments of 150-200 mL, and the DPBS is collected in a sterile bottle warmed to body temperature (Gomes et al., 2014). After collection, the bottle containing flush medium is kept warm until the embryos can be washed, counted, sorted and graded based on their quality with the aid of a dissecting microscope. However, many researchers and producers use an in-line embryo filter, which filters embryos as they are collected. Sexing Technologies Quality ET Services (2012) reports embryos are non-surgically recovered 6-8 d after breeding through the flush of the uterus via a collection line and are cached in a filter for later search and grading. After grading, the embryos are loaded into straws with either freezing

or transfer medium for storage in liquid nitrogen for future transfer to a recipient or transfered immediately to an estrus-timed recipient, respectively (Hesser et al., 2011). It is important to ensure the embryo and the recipient's uteri are at the same stage of the estrous cycle.

Cows superovulated over the course of four successive estrous cycles have been reported to still have degenerating corpora lutea on their ovaries from the previous superovulation (Lubbadeh et al., 2014), so it is important to take this into consideration when palpating the ovaries of a cow in such a circumstance so as not to overestimate the current superovulatory response.

Variations on Basic Synchronization and Superovulation Protocols

Estrous-synchronizing cattle is an important precursor to superovulation. Another basic estrous synchronization protocol involves an injection of GnRH on Day 0 to ovulate/luteinize most large follicles present on the ovaries (DeJarnette et al., no date). This induces a new follicular wave to begin one to two days later. Seven days after the GnRH injection, a $PGF_2\alpha$ injection is given to induce a more synchronous heat, as more cows will possess mature dominant follicles of similar size.

There are several variations on this basic synchronization protocol described above. One variation, known as Ovsynch, adds a second GnRH injection 48 h after the $PGF_2\alpha$ injection, which induces ovulation of the dominant follicle recruited after the first GnRH injection (DeJarnette et al., no date). This allows animals to be inseminated 8-18 h after the second GnRH injection, eliminating the need for heat checking, although it is still recommended. Ovsynch is used mainly by dairy farms, whereas another variation, called CO-Synch, is popular in beef herds. CO-Synch involves the same injections and

timing as Ovsynch, but animals are bred at the same time as the second GnRH injection is given, eliminating one animal handling period and making the protocol more feasible for a beef producer. Another variation, called SelectSynch, is a great option for producers who like to breed their animals at a standing estrus. Cows are either bred at estrus detection for 3-5 d after being given $PGF_2\alpha$ or bred to estrus for 72 h after heat detection, with animals showing no heat being given a second GnRH injection at the time of breeding, 72 h after $PGF_2\alpha$ administration. SelectSynch also has an economic benefit, as only cows which are not observed in estrus receive a second GnRH injection. A final option, Presynch, synchronizes cows to the early stages of the estrous cycle, allowing optimum response to GnRH. Two $PGF_2\alpha$ injections are given fourteen days apart, with the second being given 12-14 days before any $PGF_2\alpha$ or GnRH injections are given corresponding to one of the above protocols.

Inserting an EAZI-BREED CIDR into the cows' vaginas is another way to synchronize estrus (DeJarnette et al., no date). The CIDRs can be inserted at any random stage of the estrous cycle. Seven days after insertion, the CIDR is removed and many open cows will return to estrus in a synchronous manner over the next three or four days. This protocol can also be used in cases where it is unknown if the cows being synchronized are pregnant. Rathbone et al. (2002) developed the CIDR optimized for seven d of P_4 release in cattle from an existing commercially available bovine intravaginal insert.

When used in conjunction with a superovulation protocol, any of the previous estrous synchronization protocols will allow cows to come into heat together and the producer or researcher will be able to inseminate and flush the cows on the same day.

A novel synchronization and superovulation protocol, called P-36, was developed in Brazil (Baruselli et al., 2006). This protocol involves the placement of a progesterone/progestagen-releasing device (CIDR) in the cow to be superovulated, and the device is removed 36 h after $PGF_2\alpha$. Ovulation is then induced with LH administration 12 h after removing the progesterone/progestin releasing device (48 h after $PGF_2\alpha$ administration). Fixed time artificial insemination is performed 12 and 24 h after the LH injection. This protocol was tested in a commercial herd of Nelore cattle. One hundred thirty six animals were superovulated using the P-36 protocol and embryo collection was performed. The number of ova/embryos and transferable embryos and pregnancy rates following nonsurgical transfer of fresh embryos were evaluated and recorded. An average of 13 embryos was recovered from each treated cow, and 9 embryos from each cow, on average, were scored as transferable. The pregnancy rate for this study was 43.5%.

Another superovulation protocol begins with the administration of a subcutaneous $PGF_2\alpha$ implant (Synchrocept-B), followed by giving similar doses of FSH twice daily over the course of four days, beginning on the fourth day after estrus was observed (DeJarnette et al., no date). Baruselli et al. (2006) superovulated ten Nelore cows by placing them randomly into one of two groups in a cross-over experimental design and superovulating them 35 d apart with Folltropin V. Cows received a CIDR or DIB and 2.5 mg estradiol benzoate, and superovulation started on day 4. Cows received $PGF_2\alpha$ on the morning of Day 6 and CIDRs were removed on the morning (P-24) or afternoon (P-36) of Day 7. Cows superovulated with the P-36 protocol ovulated fewer total ova and fewer

total fertilized ova, but more transferable embryos than when the same cows were superovulated with the P-24 protocol.

Studies Successfully Using Folltropin-V, Fertagyl, Factrel, and Lutalyse®

Many studies have successfully used the same drugs in their superovulation protocols that were used in the present experiment, namely Folltropin-V®, Fertagyl®, Factrel®, and Lutalyse®, and these studies are described in detail in later sections of this literature review.

Folltropin V® is a porcine FSH (pFSH) preparation, which is the preferable form to use because pFSH-treated animals have more ovulations, fewer unovulated follicles, more recovered embryos and a greater number of transferable/freezable embryos compared to animals treated with eCG (Hiraizumi et al., 2015). Barati et al. (2006) used Folltropin-V® to superovulate 32 Sistani (*Bos indicus*) cows divided into three groups to receive 120, 160, or 200 mg Folltropin V®. Barati et. al (2006) observed the group receiving 120 mg Folltropin V® produced an average of 14.2 ova, whereas cows receiving 160 and 200 mg Folltropin V® produced 16.7 and 24.7 ova/embryos, respectively. Other studies have shown that it is possible to superovulate *Bos indicus* cattle using less FSH than is needed to superovulate *Bos taurus* cattle (Baruselli et al., 2006), but, as this experiment demonstrates, the more FSH a cow is given, the more ova she will produce (Barati et al., 2006).

Folltropin V® was also used in a study performed by D'Occhio and colleagues (2013), who chose it because it contains minimal LH activity. Nulliparous Zebu heifers were randomly assigned based on live weight to one of four groups: GnRH agonist-standard FSH, GnRH agonist, double dose FSH, GnRH agonist, saline FSH, and GnRH

agonist, polyvinylpyrrolidone FSH (the PVP was used to allow a slow-release of FSH). The follicular status and ovulation rates of all groups did not differ significantly from one another.

Folltropin V® was used as part of a complete superovulation protocol by Ratnaparkhi and colleagues (2014) as well, when they analyzed the effects of using two different doses of FSH to superovulate buffalo heifers. No significant difference in superovulatory response was observed between the two groups.

Superovulation studies almost always report the successful use of Lutalyse®. One such study was performed by Dias and colleagues (2010), in which Lutalyse® was used as part of a complete superovulation protocol for a project comparing two doses of GnRH, the results of which are discussed in a later section. Lutalyse® was also used by a group of researchers in India as part of a complete superovulatory protocol in buffalo heifers (Ratnaparkhi et al., 2014). It was administered to all the animals 72 h after the initiation of superovulatory treatment to induce estrus (presumably by starting with the regression of the CL, which would cause a new wave of follicular growth to occur) (Ratnaparkhi et al., 2014). Xu and colleagues (1996) also chose to use Lutalyse® in their study when they compared the efficiency of two estrus synchronization regimens.

Bordignon et al. (1997)performed a study in which they successfully used 200 μg of Factrel® during a superovulation protocol performed on 23 Holstein heifers. The rationale was they thought GnRH injections improve the oocyte recovery rate and that oocytes from cows treated with GnRH would have a higher developmental competence than those obtained from cows not treated with GnRH. This objective of this study was to determine the effects, if any, GnRH would have on the recovery rate and in vitro

developmental competence of oocytes obtained from superovulated heifers. The dominant follicle of each heifer was removed by transvaginal aspiration between days 9 and 13 of Folltropin V® treatment, injected in 8 decreasing doses 12 h apart (Bordignon et al., 1997). Luteolysis was induced with 500 μg of Estrumate® 48 h after the first FSH injection. Thirty four hours after the $PGF_2\alpha$ injection, 12 heifers were injected with 200 μg Factrel®; 60 hours after $PGF_2\alpha$ administration, all animals underwent a transvaginal follicular aspiration procedure. GnRH injections improved oocyte recovery percentage and an increased percentage of oocytes had a higher developmental competence than those obtained from non-GnRH-treated animals.

Experiments Modifying the Superovulation Protocol

Many superovulation studies use a protocol in which the dose of FSH given to a donor cow over the course of four days decreases as the days of the protocol go on (D'Occhio et al., 2013). For example, one study decreased the amount of FSH given during a superovulation protocol each day by a total of 20 mg (D'Occhio et al., 2013). Despite the huge number of bovine superovulation studies which have been performed, there is still no suggested dose of FSH to give to cattle (Barati et al., 2006). Dosages of 360-400 mg Folltropin V® is generally recommended to adequately superovulate Bos taurus cows. Bos indicus cattle can be successfully superovulated with 250-280mg of Folltropin V® (Barati et al., 2006).

Ratnaparkhi et al. (2014) compared the effects of two doses of FSH given to Berari buffalo heifers on superovulatory response. Heifers were injected with a total of 500 or 560 mg FSH, given in the form of twice daily doses over the course of four days which decreased in volume each day. All animals were given the same amount of $PGF_2\alpha$

four d after initiating the superovulatory protocol and all animals were observed in estrus. Cows given 560 mg FSH came into estrus a little earlier and remained in estrus a little longer than animals given 500 mg. No significant differences were reported in any parameters associated with the superovulatory response between the two groups.

Staigmiller and colleagues (1994) compared superovulation endpoints of two groups of 10 crossbred heifers, where one group was injected with a single pFSH injection and the other received the same amount of pFSH over the course of 8 injections given during 4 consecutive days. Heifers superovulated with a single injection of pFSH yielded fewer ovulations, ova, and transferable embryos than their counterparts receiving 8 injections. In addition, fewer unfertilized ova were collected from heifers receiving 8 total pFSH injections (Staigmiller et. al, 1994).

Baruselli et al. (2006) demonstrated that Nelore cattle, a *Bos indicus* breed, can be superovulated with less FSH compared to doses commonly used for *Bostaurus* cattle. Twenty-three Nelore cows were divided into three groups and injected with totals of 100, 133, or 200 mg FSH divided into eight injections and administered over four consecutive days. No differences were observed among groups in numbers of CL, total embryos and ova collected, transferable embryos and freezable embryos.

Barati et al. (2006) carried out a study using 32 Sistani cows (a Bos indicus breed of beef cattle) divided into three groups based on FSH dose. Cows received eight doses of Folltropin V® given over the course of four days amounting to 120, 160 or 200 mg total, and cattle were subsequently artificially inseminated at the appropriate time in the superovulation protocol. Ova and embryos were recovered seven d after cows were observed in standing estrus. The humidity, environmental and rectal temperatures were

recorded daily from -2 to 3 d of the superovulatory estrus at 8:00am, 2:00pm and 8:00pm to observe any correlations these data may have on the number and quality of ova and embryos recovered. The same experimental protocol was carried out both in summer and winter. Barati et. al (2006) reported a correlation between dose of FSH and the number of CL with 160mg FSH being the most effective. Most cows receiving 200 mg FSH came into estrus at 8:00am, whereas cows receiving 160 mg FSH were observed in estrus at 2:00pm and cows receiving 200 mg FSH were in estrus at 8:00pm on the seventh day after protocol initiation. Cows had noticeably lower rectal temperatures the day after they came into estrus, and a correlation was observed between rectal and environmental temperatures in winter. No differences in the number of ova or embryos collected were observed between winter and summer.

Kimura et al. (2007) set out to determine if Japanese beef cows could be successfully superovulated after one intramuscular injection of pFSH adsorbed in aluminum hydroxide gel was administered in the gluteal muscle. Aluminum hydroxide gel has been shown to adsorb macromolecules (including toxoids and proteins) and release them in small doses over a period of time, making it a popular vector for vaccinations. No significant differences in numbers of CL, large follicles, embryos collected, or transferable embryos were observed between cows superovulated with one injection of 30 mg pFSH or 8 injections of decreasing doses of pFSH for a total of 30 mg over the course of four days.

D'Occhio et al. (2013) administered two different doses of FSH to Zebu heifers. One group received a total of 168 mg while the other group received 336 mg over four

days. No significant difference in the number of follicles present on the ovaries was found between the two groups.

Dias et al. (2010) superovulated sixteen heifers with either 100 or 200 µg GnRH, the same doses examined in our study. Dias et al. (2010) hypothesized the higher dose of GnRH (Cystorelin) would be more effective in inducing a surge of LH, which would be amplified by the administration of exogenous E_2. It was expected the release of more LH would increase the ovulation rate in cows receiving more GnRH. Although the increased GnRH dose induced a larger amount of LH during theLH surge, ovulation rate did not differ from the 100 µg group. Contrary to Dias et al. (2010), Mihm et al. (1998) observed a greater ovulation rate in heifers treated with 200 vs 100 µg GnRH.

Variability In Ovarian Response To Superovulation

Numerous factors contribute to the variable ovarian responses observed when a cow is superovulated, but perhaps the biggest factor is follicular wave status (Bo et al., 1995). This is why such pains are taken to physically or hormonally remove the dominant follicle from a cow's ovary prior to starting a superovulation protocol, as removal of the dominant follicle ensures the absence of inhibin secretion and allows a new wave of follicular recruitment to occur (Bo et al., 1995). Removing the dominant follicle also ensures greater estrous synchrony between the donors and recipients.

One factor thought to be responsible for the variable superovulatory response was the presence of small quantities of LH present in FSH preparations obtained from porcine pituitary extracts (Sendag et al., 2008). Removal of LH from these pituitary preparations of FSH has been found by several researchers to reduce the variability in superovulatory response, without diminishing the positive effect of FSH on follicular growth (Sendag et

al., 2008). According to Hesser et al. (2011) an FSH preparation used for superovulation should not contain more than 15-20% detectable LH activity, although the exact ratio could also depend on the individual's age, parity, and overall ovarian function.

Kimura et al. (2007) looked at the effects of superovulating cows every two to three months for a year. For each trial, cows were detected in estrus on the morning of the fourth day after initiation of superovulatory treatment. For the first, second, third, fourth, and fifth rounds of superovulation, the average number of corpora lutea were 12.4, 13.8, 9.0, 9.8 and 12.0, respectively. Likewise, total ova recovered were 12.0, 12.6, 6.8, 7.6, and 11.4, and transferable embryos were 11.4, 10.4, 6.6, 4.8, and 10.4, respectively. No significant differences between rounds of superovulation were observed for any of these end points.

Weather may also be the cause of variability in superovulation response (Barati et al., 2006). Season has an extreme impact on superovulatory response of *Bos taurus* cattle, however no effect was observed on a contemporary group of *Bos indicus* cattle in summer and in winter. No effect of season was observed on the superovulatory response of these cows (Barati et al, 2006).

Age is another factor which may impact superovulatory success. Aged donors (13-16 years of age) are known to exhibit a lower follicular and ovulatory response when superovulated (Hesser et al. 2011). Other factors contributing to variable superovulatory responses can be attributed to the farm where the animals are maintained, and may be related to environmental location, animal management, semen and embryo handling and nutrition (Hesser et al., 2011). For example, Yaakub and colleagues (1998) reported that

increasing the concentrate intake from 3 kg to ad-libitum consumption reduced the superovulatory response and yield of good quality embryos in heifers.

First-Time Superovulations

Several studies have reported using superovulation protocols on peripubertal heifers. Ax et al. (2005) evaluated the efficacy of performing superovulation and embryo recovery procedures on heifers at 7.8 months of age and any impacts these procedures may have on future reproductive performance. Ten peripubertal heifers were superovulated and only nine responded. Twelve total embryos were recovered, seven were transfered to recipients, and five resulted in pregnancies. Ax et al. (2005) observed that these peripubertal heifers produced 122 kg less milk, calved 7-10 d later, and conceived 14 d sooner than their full-siblings which were not peripubertally superovulated.

Dias et al. (2010) superovulated 14-18 month old nulliparous heifers in a study evaluating different concentrations of GnRH and P_4. Heifers were assigned to one of four groups: low P_4 (received 50 mg $PGF_2\alpha$ given in two injections), 200 µg GnRH; low P_4 (received 50 mg $PGF_2\alpha$ given in two injections), 100 µg GnRH; high P_4 (no $PGF_2\alpha$), 200 µg GnRH; and high P_4 (no $PGF_2\alpha$), 100 µg GnRH. Seven out of eight heifers which had had their CL removed to decrease P_4 (low progesterone group) and received 200 µg of GnRH (high GnRH) ovulated, and all eight heifers in the low progesterone group which had received 100µg GnRH (low P_4, low GnRH) ovulated. However, only 1 heifer in the group of 8 which retained their CLand received 100µg GnRH(high P_4 group, low GnRH) ovulated, and 5 out of the 8 heifers in the high P_4 group who received 200µg GnRH (high P_4, high GnRH) ovulated. In addition to supporting the hypothesis that increased GnRH,

in combination with low P_4, may increase the ovulation rate of superovulated animals, these authors also demonstrated that younger animals can be successfully superovulated (Dias et al., 2010).

Causes For Production of High Numbers of Degenerate Embryos

Aged donor cows tend to not produce as many follicles or ovulate as many oocytes during superovulation as their younger, less superovulated counterparts, and it is speculated one reason could be ovarian hyperstimulation (Hesser et. al, 2011). After analyzing multiple studies Desaulniers et al. (1995) concluded that reproductive disorders not visible at the start of a superovulation protocol may be a contributing factor. Desaulniers et al. (1995) observed higher estradiol concentrations during the follicular phase in cows yielding more embryos and ova as compared to their less productive peers. The less productive donors also exhibited a post-ovulatory increase in FSH. Desaulniers et al. (1995) suggested these phenomena are at least partly responsible for the low superovulatory response displayed by some donors. Cows older than 10 years of age also possess more atretic follicles than younger herdmates. Because superovulation allows early atretic follicles to ovulate and be fertilized it is thought these atretic follicles are the source of the high number of degenerate embryos obtained from older donors. Vieira et al. (2013) compared superovulatory responses between cows and heifers and found that while older animals had more CL and produced more ova and embryos than younger animals, heifers had a higher fertilization rate and produced more transferable embryos. Vieira et al. (2013) also observed embryos recovered from older donors were more likely to survive once transfered to a recipient than those taken from heifers.

It is widely accepted that the cause of the high number of degenerate embryos and unfertilized ova (UFO) commonly encountered when a cow is superovulated are due to the hormones given during the superovulation protocol (most notably FSH) causing the ovulation of atretic follicles. The size of follicles ovulated during superovulation can be smaller than those ovulated during a natural estrous cycle (Desaulniers et al., 1995), and it is speculated nonviable embryos originate from such follicles as these were prevented from undergoing atresia by superovulation. Blondin et al. (1996) superovulated twenty-two heifers using 4, 6, or 8 4-mg FSH injections given twice daily throughout d 8-12 of the estrous cycle (corresponding to Days 8-12 when Day 0 is the day an injection of Lutalyse® is given to restart an animal's estrous cycle). Blondin et al. (1996) found that although cows which were superovulated had lower atresia rates and produced more oocytes and embryos than cows which were not superovulated, the developmental competence of oocytes from superovulated cows was lower than that of non-superovulated cows. Blondin et al. (1996) suggested the cause of this phenomenon is the absence of oocyte exposure to pre-ovulatory and atretic environments, because oocytes from superovulated cows only encounter exposure to a prolonged rapid growing phase as they develop. This does not prevent low-quality or atretic follicles from ovulating. Detterer et al. (1997) also reported that cows which have had two or more calves produce more total embryos than heifers or cows which have had only one calf, but the number of transferable embryos is not significantly greater compared to heifers or cows with one calf.

Other causes for suboptimal numbers of ova and embryos collected from cattle include combinations of genetic, environmental, and management factors (Nivet et al.,

2012). Tonhati et al. (1998) set out to determine to what extent a genetic link may affect superovulatory responses of cows and their female offspring. Additionally, a heritability coefficient (the probability that genes related to superovulation will be passed from a cow to her female offspring) has been developed by Tonhati et al. (1998). This heritability estimated was developed based on data collected from 2724 superovulations performed on 1580 cows, and was applied to an extensive data set (5387 superovulations of 2941 cows inseminated with semen from one of 690 bulls). Calculated heritability of an acceptable superovulation response was 0.13. Tonhati et al. (1998) concluded that environmental factors play a major role in a cow's superovulation response, and there is little evidence suggesting future superovulatory responses of a particular animal can be predicted based upon past superovulatory responses. Repeatedly superovulating the same donor may also play a part in decreasing their superovulatory response (Tonhati et al., 1998). Tonhati et al. (1998) also derived a repeatability coefficient. Estimation of the repeatability coefficient for an acceptable superovulatory response is 0.03. The repeatability coefficient was developed based on data from the data set used to generate the heritability coefficient.

Studies have shown exposure to hormones used in the superovulation protocol cause a cow to build up resistance to the hormones (Blondin et al., 1996). D'Occhio et al. (2013) distributed GnRH agonist implants to cows six months before commencing a superovulation protocol. Heifers which received GnRH agonist implants had fewer gonadotropes in their anterior pituitary glands, and lost pulsatile LH secretion and the transient increase in FSH production. The absence of adequate plasma concentrations of these hormones arrested the growth of follicles in the treated animals at the antral follicle

stage. However, after four days of FSH injections given at the appropriate times and in sufficient doses, follicular responses of cows treated with a GnRH agonist implant did not differ from the control cows.

Kanitz et al. (2002) observed as an animal is given more FSH, the number of ovulated oocytes increases as a function of the amount of FSH given, but that it eventually plateaus. These findings correlate somewhat with Barati and colleagues (2006) who found overstimulating Sistani cattle with 250mg of FSH resulted in a high incidence of anovulatory follicles.

Lubbadeh et al. (1980) repeatedly superovulated the same group of cattle four times. The first three superovulations were performed back-to-back; that is, during three successive estrous cycles. The animals were allowed to undergo one "normal" estrous cycle between the third and fourth superovulation treatments. Ovulations for the four superovulation periods averaged 17.9, 15.8, 10.9 and 21.5, respectively. Neither the decreased number of ova ovulated in rounds 2 and 3 nor the increased number of ova ovulated in round 4 were statistically significant compared to any of the other rounds.

Cost Effectiveness of Superovulation

The most labor-intensive and expensive part of the superovulation protocol is associated with FSH (Hesser et. al, 2011). Currently, it costs between $150-200to purchase the FSH, GnRH, $PGF_2\alpha$ and CIDRs necessary to superovulate one cow or heifer, and more than $150 just to purchase the FSH alone (Gomes et al., 2014). Greer et al. (1992) reported a major limitation in the application of embryo transfer technology to production of domestic animals is obtaining a large number of embryos at a reasonable cost.

Hesser et al. (2011) proposed the use of recombinant hormones and alternative methods of delivery may decrease the cost associated with superovulation, providing an economic alternative to the use of eight injections of pituitary-derived FSH. Traditionally, superovulation has used pituitary extracts containing gonadotropins to obtain the hormones necessary for the protocol, including FSH and recombinant GnRH (Hesser et. al, 2011). The disadvantage of recombinant gonadotropins is their high cost (Gerli et al., 2004, Hesser et al., 2011), but their use offers three advantages over their naturally derived counterparts, which are: less LH contamination in FSH preparations, fewer batch inconsistencies, and a reduced risk of transferring infectious agents, including prions (Hesser et. al, 2011), the last of which can also be reduced by using pFSH preparations. Industrializing the production of recombinant hormone preparations will also substantially lower the production costs as the primary contributing factors are culture media and labor (Hesser et al., 2011). Recombinant gonadotropins are produced in vitro using Chinese hamster ovary (CHO) cells cultured in a medium with fetal bovine serum (FBS) (Hesser et. al, 2011). The hormones being produced are secreted into the FBS medium, from which they are extracted and purified (Hesser et. al, 2007) before being made available for purchase.

Recombinant FSH (rFSH) cost in the superovulation protocol can be offset by high numbers of offspring, particularly female offspring, being generated by the bovine donors (Li et al., 2009). One way of achieving this goal is immunizing donors undergoing the superovulation protocol against inhibin and inseminating donors with sex sorted semen (Li et al., 2009). In 2009, Li et al. superovulated 28 Holstein heifers with the objective of maximizing embryo yield and quality produced by superovulation. Cows

were divided into two groups. One group would receive a single 1 mg injection of rinhibin immunogen followed by two 0.5 mg booster injections of the same substance, while the other group received an initial injection of 0.5mg of recombinant inhibin immunogen and two 0.25mg booster injections of the same protein (Li et al., 2009). All cows were inseminated using sex-sorted female spermatozoa at 0 and 8 hours after observation of estrus (Li et al., 2009). No statistically significant differences between the control, high dose, or low dose groups in regards to number of degenerate embryos or unfertilized ova were observed however animals in the high and low dose groups produced significantly more total and transferable embryos than the control group. The high dose group also had more unfertilized ova than the low dose and control groups (Li et al., 2009).

Another way to reduce the cost of hormones associated with superovulation is to simply use lower hormone doses. *Bos indicus* breeds of cattle, such as Nelore, can be superovulated using less FSH than *Bos taurus* breeds of cattle, such as Angus and Holstein (Baruselli et al., 2006). To this end, there are several researchers carrying out protocols on *Bos taurus* breeds of cattle in which the dose of FSH given each day is smaller than that given on the previous day (Hiraizumi et al., 2015).

Development of a Dipstick Style Assay to Assess Embryo Viability and Predict Success of Pregnancy Establishment

Fertilization and Hatching

Defining the day of estrus as Day 0, fertilization occurs in the ampullary-isthmic junction of the cow's oviduct on Day 1 (Betteridge and Flechon, 1988). Twenty-four to

twenty-eight hours after ovulation, the first cleavage division of the zygote, into 2 blastomeres, occurs, and the second division, into four blastomeres, occurs on Day 2. In mammals, these divisions which occur early on are asynchronous, and the cells which divide earlier make up the inner cell mass (ICM), which will eventually become the embryo. These cell divisions are also unequal, so that one daughter cell is bigger than the other, and larger cells become "outside" cells while smaller ones become "inside" cells, and it is during these divisions that a difference between the cells which will become the embryo and those which will become the trophoblast can be seen.

As the bovine embryo develops, it is referred to by the number of cells it contains until day 5, when, with 16 cells or more, it becomes difficult to determine the number of cells (Betteridge and Flechon, 1988); 16 cell bovine embryos are called morulae, so named because of their resemblance to mulberries. The borders of blastomeres become faint as the embryo becomes a 32-cell embryo as they undergo compaction, and the embryo becomes a compact morula. Once compaction has been completed, a fluid-filled cavity called a blastocoele begins to form, usually around day 7. Bovine embryos can hatch out of the zona pellucida surrounding them as early as day 7.

After hatching, the blastocyst re-expands and the ICM is now referred to as an embryonic disc (Betteridge and Flechon, 1988). This embryonic disc is still covered with trophectoderm until day 12, at which point the hatched blastocyst becomes more oval rather than spherical in shape after a hole in Rauber's layer of trophectoderm has formed. Just before a hole in the trophectoderm layer forms, the cells of the ICM differentiate into embryonic ectoderm, mesoderm, and endoderm. Elongation of the embryo starts between d 12 and 14, and maternal recognition of pregnancy occurs on day 16-17.

Plasminogen Activator and its Applications to Bovine Embryo Transfer

Urokinase-type plasminogen activator (uPA) is a serine protease which cleaves the proenzyme plasminogen into the active enzyme, plasmin. uPA is associated with bovine blastocysts shedding the zona pellucida (Kaaekuahiwi and Menino, 1990), a process all normally developing bovine embryos must go through so they can implant, elongate, and develop to term. The quantity of uPA produced by a bovine blastocyst can be a useful marker of embryo viability, and can be an indicator of which embryos are more likely to implant and generate a pregnancy than an embryo producing little or no uPA. Hatched embryos are not usually transfered to a recipient, as it is more preferable to transfer a viable embryo before it hatches. Also, costs could be reduced because the number of females failing to become pregnant because marginal embryos were transfered would be reduced, and a precise, reliable uPA assay would allow only the most vigorously developing embryos to be selected for transfer. Currently, there is no commercial test to determine the presence or quantity of uPA in embryo culture medium, and embryos are chosen for transfer based solely on a subjective grading scheme.

Plasminogen Activator and the Bovine Embryo

A caseinolytic agar gel assay was used by Menino and Williams (1987) to determine uPA production in bovine embryos cultured through hatching. Embryos were collected from estrus synchronized and artificially inseminated superovulated cows and cultured in Ham's F-12 medium supplemented with 15 mg/mL bovine serum albumin (BSA) containing 0, 15, 30, 60, or 120 µg/mL human plasminogen in a humidified atmosphere of 5% CO_2 in air at 37° C. Cultures were observed and stage of development

recorded daily. Medium was also collected daily, starting at the commencement of the protocol and continuing through 288 h of culture. uPA and plasmin levels in the culture medium were determined using the caseinolytic assay. No significant effect on the number of embryos developing to initiating hatching blastocyst, hatched blastocyst, attached blastocyst and attached blastocyst with trophoblastic outgrowth stages was observed with the addition of plasminogen to the culture medium, but hatching initiation and completion were accelerated as more plasminogen was added. Menino and Williams (1987) suggested a sublysis of the zona pellucida may be caused by uPA converting uterine plasminogen to plasmin thereby facilitating the hatching process.

Kaaekuahiwi and Menino (1990) evaluated the relationship between uPA production and in vitro bovine embryo development. Embryos in the late morula to blastocyst stages were collected from fifteen crossbred beef cows. Embryos were morphologically evaluated using the grading system described by Lindner and Wright (1983) and were cultured individually in 25 µL microdrops of Ham's F-12 with 15 mg/mL BSA under paraffin oil in a humidified atmosphere of 5% CO_2 in air at 37° C for 6 d. At the beginning of culture and every 24 hours afterward for 6 d, developmental stage, embryo diameter and zona pellucida thickness were determined, with the diameter prior to hatching including the zona pellucida, and every 24 h, embryos were transfered to fresh microdrops. Culture medium (15 µL) was recovered and frozen at -20 C until it could be assayed for uPA. As a control, culture medium from drops not containing embryos were also recovered and frozen. After culture, embryos were recovered from microdrops, washed twice in 2.0% sodium citrate, transfered to 0.7% sodium citrate for 3

min, fixed onto microscope slides with 25% acetic acid in ethanol, air dried and stained with hematoxylin and eosin. Kaaekuahiwi and Menino (1990) determined the concentrations of PA in the culture medium using a caseinolytic assay (Menino and Williams, 1987). To quantify uPA production, 15 µL of 120 µg/mL human plasminogen as the substrate was combined with 15 µL of either recovered culture media or 0, .0009, .0045, .0091, and 0.04455 mU of urokinase standard and incubated for 15 mins at 37° C. After incubation, 25µL of incubated mixture were pipetted into 4 mm diameter wells cut in casein-agar gel plates and incubated at room temperature for 24 h. Casein-agar gel plates were fixed for 15 min with 3% acetic acid and rinsed in tap water, and the diameters of the caseinolytic zones were measured with an electronic digital caliper. PA concentrations were determined by comparing standard curves of caseinolytic ring diameter by log urokinase concentration, and the amount of PA produced by the embryo was determined by subtracting the amount of PA in the medium without an embryo from the amount in the medium with an embryo for the 24-h interval. Kaaekuahiwi and Menino (1990) found that total PA production was correlated positively with embryonic size, developmental stage and cell number, and negatively correlated to zona pellucida thickness. More PA was produced by hatched embryos than by those which did not hatch, and the authors stated that whether embryonic PA production can be used as a means to predict success in pregnancy establishment remains to be determined.

Existing Embryo Grading Schemes

Currently, there are two commonly used grading schemes available for determining the quality of a bovine embryo destined for transfer, one method grades transferable embryos on a scale from 2-4, with 4 being the best quality (a grade of 1

describes an unfertilized ovum) (Shea, 1981). Another grading scheme utilizes four ranks: poor, fair, good and excellent, to determine the likelihood of a transfered embryo generating and maintaining a pregnancy (Lindner and Wright, 1984). Both of these schemes are based solely on observation of gross morphology where embryos just prior to transfer are evaluated microscopically for flaws, e.g. degenerate blastomeres, vesicles, etc. Results using these grading schemes are variable; 71% of grade 4 embryos and 45% of excellent quality embryos generated pregnancies following transfer. The major drawbacks of currently existing grading embryos are that they are subjective and dependent on previous embryo grading experience (Renard et al., 1980).

Development of Embryo Viability Assays

A handful of embryo viability assays have been developed, but all have the disadvantage of not being readily available to a practitioner working in an "on-farm" environment. One technique that has been used for decades is to culture all questionable embryos for 24 h to see if they develop any further (Shea, 1981). Bovine embryos respond well to culture and still result in a high pregnancy rate after 24 h. Shea (1981) also noted embryos which have not hatched yet respond best to culture.

Renard and colleagues (1980) measured glucose uptake as an indicator of in vitro bovine embryo viability. Blastocysts were recovered via a cervical technique from Charolais heifers ten or eleven d after the heifer had been seen in estrus, and were morphologically examined in B2 medium. After examination, blastocysts were transfered to sterile glass tubes containing 0.5 mL B2 medium with 600 μg glucose, gassed with 90% N_2 , 5% O_2, 5% CO_2 and cultured at 37° C for 20 h. After the culture period, 48 out

of 59 embryos had increased in size, and 25 of these 48 showed a significant uptake of glucose from the culture medium. Of all the transfered embryos which took up a significant amount of glucose, 69.2% generated pregnancies, whereas 40.7% of the transfered embryos which did not take up a significant amount of glucose generated pregnancies. The pregnancy rate for directly transfered blastocysts of the same age as those being cultured was 50%.

Hoppe and Bavister (1983) evaluated whether a correlation existed between fluorescent dye binding and an embryo's ability to continue developing. Two groups of hamster 8-cell embryos were used: (1) freshly collected embryos and (2) cultured embryos which failed to develop. A stock solution of fluorescein diacetate (FDA) was prepared by dissolving 5 mg FDA/mL acetone. The final solution was made by adding 0.5 µL of stock FDA solution to each mL of holding medium (TALP-Hepes or PB1). Each embryo was incubated in this solution for 1 min, rinsed twice for 1 min each in fresh medium and transfered to a glass depression slide in a small amount of medium and exposed immediately to UV light for 15 seconds. If embryos fluoresced through a range of intensities, they received a positive score; if embryos did not fluoresce they received a negative score. A significantly greater proportion of freshly recovered embryos fluoresced in the FDA assay than did the cultured embryos.

Polgar and colleagues (1994) evaluated whether a fluorescence photobleaching recovery (FPR) technique can be used to evaluate embryonic viability. Oocytes, zygotes, or two-cell embryos were obtained from superovulated CF1 mice post mortem. S75, a monoclonal IgG antibody, has been shown to react with a glycoprotein found on the surface of preimplantation mouse embryos. In this experiment, fluorescein-5-

isothiocyanate (FITC) was used to label S75, and oocytes, zygotes and 2-cell embryos were washed and suspended in Whitten's medium before being mixed with one microdroplet of FITC-S75 solution for 3 mins. Oocytes, zygotes, and two-cell embryos were all washed 5 times in Whitten's medium and 20µL microdrops containing 20 µL of Whitten's medium and 3-5 oocytes, zygotes or 2-cell embryos were transfered to a microscope slide. Each embryo was exposed to a brief, intense photobleaching pulse (110 µW for 100 milliseconds) followed by periodic low intensity measuring pulses (10 µW for four milliseconds) used to recover fluorescence over a period of 50 seconds. Amplification and intensity settings were adjusted so that the signal given off by unlabeled cells was 1% of that given off by labelled cells. It was noted that degenerate embryos had a significant decrease in the glycoprotein fractional mobility of S75 labelled proteins, suggesting that degenerating zygotes either rapidly internalize S75 labelled proteins or are not competent enough to prevent S75 mAb from entering into the cytoplasm.

Developing a Dipstick Style Assay

Paek et al. (2000) described how to develop a rapid one-step immunochromatographic assay using two antibodies which bound distinct epitopes of an antigen. One antibody, labeled with colloidal gold (a signal generator) was placed in a dehydrated state on a glass fiber membrane, and the other antibody was immobilized on a nitrocellulose membrane. Three membranes were attached to a plastic substrate in the following order from the bottom: glass fiber membrane with the labeled antibody, nitrocellulose membrane with the immobilized antibody and cellulose membrane, to aid with wicking, and the membranes were cut into strips. The bottom ends of each of the

strips (the ends with the glass fiber membrane) were placed into solution containing the analyte, and the solution was wicked up each strip. The aqueous medium dissolved the labelled antibody on the glass fiber membrane and the subsequent antigen-antibody complex moved up to the nitrocellulose membrane containing the immobilized "capture" antibody. The complex reacted with this "capture" antibody and generated a signal proportionate to the concentration of analyte in the solution. Although this technology has been used for years in the production of pregnancy tests for humans, to date, no one has attempted to develop such a test for assessing embryo viability prior to a transfer.

CHAPTER 3

MODIFYING THE HORMONE STRATEGY FOR SUPEROVULATING DONOR COWS TO REDUCE DRUG COSTS WITHOUT DECREASING THE NUMBER OF HIGH-QUALITY TRANSFERABLE EMBRYOS RECOVERED

Abstract

The objective of this research was to adjust the hormone doses used in a superovulatory protocol to where drug costs can be reduced while retaining recovery of a satisfactory number of high quality transferable embryos. In the first experiment, 24 crossbred beef cows from the Oregon State University Beef Cattle Ranches were assigned to one of four treatments: 1) 400 mg FSH and 100 µg GnRH (traditional dose), 2) 400 mg FSH and 200 µg GnRH, 3) 200 mg FSH and 100 µg GnRH, or 4) 200 mg FSH and 200 µg GnRH. Embryos were collected nonsurgically 7 d after estrus onset and scored for developmental stage and quality. Dose of GnRH had no significant effects on the average numbers of ova, embryos and transferable embryos recovered, nor did it have a significant effect on percent embryos and transferable embryos recovered. However, twice as many ova were recovered from cows treated with 400 compared to 200 mg pFSH, but the percentage of transferable embryos of the total number of ova recovered was greater with 200 compared to 400 mg FSH. The high doses of both FSH and GnRH increased the number of unfertilized ova recovered. Increasing the GnRH dose was not cost effective. The objective of this second experiment was to evaluate in vitro development and plasminogen activator (PA) production by embryos recovered from cows dosed with 200 or 400 mg FSH. Twelve crossbred beef cows from the Oregon State University Beef Cattle Ranches were assigned to one of two groups: 1) 400 mg FSH and 200 µg GnRH or 2) 200 mg FSH and 200 µg GnRH. Embryos were collected

nonsurgically on d 7, scored for developmental stage and quality and cultured in Ham's F-12 containing 15 mg/mL BSA to ascertain uPA production. Dose of FSH had no significant effects on the average numbers of ova, embryos or transferable embryos recovered, nor did it have an effect on the percent embryos and transferable embryos recovered. However, cows superovulated with 400 mg FSH produced more unfertilized ova ($P = 0.08$) than cows superovulated with 200 mg FSH. More ($P < 0.05$) embryos collected from cows superovulated with 200 mg FSH hatched and PA production was greater ($P < 0.08$) cows superovulated with 400 mg FSH. Taken together, these data suggest the lower dose of FSH generated embryos which may have a greater likelihood of pregnancy establishment.

Introduction

Embryo transfer is an applied reproductive technology used to improve herd genetics and female reproductive efficiency and propagate offspring from elite sire-dam matings (Bó and Mapletoft, 2013). Although the technique is commonly referred to as "embryo transfer", the transfer part is only the latter half of the procedure. The equally critical part of an embryo transfer is the collection of embryos from superovulated donors. Because cows usually ovulate one ovum per estrous cycle, donors are commonly treated with a 4 day regimen of FSH, a pituitary gonadotropin. Costs associated with FSH are high and currently it costs at least $175 to superovulate one donor. Also, overstimulation of the ovaries by FSH occurs frequently and is counterproductive to the numbers of total and transferable embryos recovered. Further, the amount of FSH needed to induce an acceptable superovulatory response is unknown. Often practitioners will administer a second hormone, GnRH, when the donor first exhibits heat to increase the

number of ova ovulated, thereby increasing the total number of embryos produced and collected from a donor. Despite the prevalence of GnRH use in these protocols, no clear benefit of the GnRH injection on embryo production has been realized. It may be that in our attempts to increase ovulation rate, we are actually under-dosing donors with the current GnRH dosing strategy. Therefore, the objective of this study was to evaluate the number and quality of embryos recovered from donor cows superovulated with reduced dosing of FSH and increased dosing of GnRH. We hypothesized the reduced dose of FSH and increased GnRH would yield fewer total ova but a higher percentage of transferable embryos.

Materials and Methods

Experiment 1

Animals

Twenty-four crossbred beef cows which had never been superovulated were randomly assigned to one of four treatments: 1) 400 mg FSH and 100 µg GnRH (traditional dose), 2) 400 mg FSH and 200 µg GnRH, 3) 200 mg FSH and 100 µg GnRH, or 4) 200 mg FSH and 200 µg GnRH. Cattle were housed at Soap Creek Ranch and transported to Oregon State University's Steer-A-Year barn in groups of four. All cows were fed hay and had ad libitum access to water. All procedures were carried out in accordance with the Institutional Animal Care and Use Committee.

Estrous Synchronization and Superovulation

Cows were injected with 25 mg prostaglandin$F_{2\alpha}$ ($PGF_{2}\alpha$; Lutalyse®, Pfizer, Inc., New York, NY) on Day 0 (where Day 0 is the day of $PGF_{2\alpha}$ injection) to synchronize

their estrous cycles. On Days 10, 11, 12, and 13 cows wereinjected twice daily with FSH (pFSH; Folltropin -V®, Bioniche, Belleville, ON). Cows receiving the 400 mg dose of FSH were injected with 50 mg twice daily and cows receiving the200 mg dose received twice daily injections of 25 mg FSH. (On day 13 a 12.5 mg injection of $PGF_{2}\alpha$ was administered to each animal). At onset of estrus or 36 h after the last $PGF_{2\alpha}$injection of the protocol, cows received either 100 or 200 μg GnRH (Fertagyl®, Intervet Inc., Roseland, NJ).

Artificial Insemination

Twelve hours after the last $PGF_{2}\alpha$ injection, all cows were heat-checked for thirty minutes every 12 h. Cows were artificially inseminated with a single 0.5 cc straw of frozen-thawed bull semen 0, 12, and 24 h after being observed in standing heat. Cows not observed in heat were bred 36 h after the last $PGF_{2\alpha}$ injection, and 12 and 24 h later. Cows were injected with their respective dose of GnRH (100 or 200 μg) at the time of the first artificial insemination.

Nonsurgical Uterine Flush

Seven days after standing estrus, a nonsurgical uterine flush was performed on each cow using Dulbecco's phosphate-buffered saline (DPBS) with antibiotics, antimycotics, and 0.2% HTFCS in order to recover embryos. Cows were given a 0.5 cc injection of acepromazine in the coccygeal vein and 3.0-5.0 mL of lidocaine was administered as an epidural to every animal. A Foley catheter was passed through the cervix and into the uterine body, and the balloon was filled with 15-20 cc of air so the catheter would not exit the uterine body during the flush. The uterus was repeatedly filled

with and emptied of flush medium, which was collected into a bottle warmed to body temperature. A total of 1000 mL of medium was used to flush each cow. Flush medium was transported to the laboratory in the collection bottle and poured through a Teflon mesh to recover ova. Ova were recovered from the screened medium by aspiration and transfered to 25 μL microdrops of Ham's F-12 + 1.5% BSA for embryo grading.

Embryo Grading

Total numbers of ova recovered from each cow were recorded and classified as embryos or unfertilized ova. Embryos were scored for development and morphology according to the grading scheme described by Lindner and Wright (1983), and further classified as transferable or non-transferable.

Experiment 2

Animals

Twelve crossbred beef cows which had never been superovulated were randomly assigned to one of two treatments: 400 mg FSH and 100 μg GnRH or 200 mg FSH and 100 μg GnRH. Animals were housed at Soap Creek Ranch and transported to Oregon State University's Steer-A-Year barn in groups of four. All cows were fed hay and had ad libitum access to water.

Cows were estrus synchronized, superovulated, and artificially inseminated andembryos were collected nonsurgically and scored as described in Experiment 1. Ninety to 120 d after the first round of superovulation, cows were superovulated a second time using the same dosing strategy as in the first round. All procedures were carried out in accordance with the Institutional Animal Care and Use Committee.

Embryo Culture

Good to excellent quality late morulae to blastocysts were cultured, one embryo per 15 µL drop,in Ham's F-12 + 1.5% BSA for 8 d. At 24 h intervals embryos were evaluated for viability and overall development and transfered to fresh micro-drops. Conditioned medium (10 µL) was recovered for quantifying plasminogen activator. Plasminogen activator is an enzyme produced by vigorously developing embryos and is correlated with embryo cell number and development to advanced cell stages (Kaaekuahiwi and Menino, 1990). Embryonic plasminogen activator production was determined via a caseinolytic agar gel assay with human urokinase as the standard (Menino and Williams, 1987).

Statistical Analysis

For Experiment 1, differences in total numbers of ova, embryos, transferable embryos, and unfertilized ova as well as the percent embryos, transferable embryos, and unfertilized ova of the total ova recovered were analyzed using analysis of variance (ANOVA) for a 2 x 2 factorial design, where the sources of variation were dose of FSH, dose of GnRH, and the FSH x GnRH interaction. If significant effects were observed in the ANOVA, differences between means were evaluated using Fisher's least significant differences procedures. All analyses were performed using the NCSS statistical software program.

For Experiment 2, differences in total numbers of ova, embryos, transferable embryos, and unfertilized ova as well as the percent embryos, transferable embryos, and unfertilized ova of the total ova recovered were analyzed by repeated measures analysis of variance (ANOVA) where the sources of variation were FSH dose (200 or 400 mg), superovulation round and the FSH x round interaction. Differences in the times required

for embryos to reach a particular cell stage and PA production by cultured embryos were analyzed by repeated measures ANOVA where FSH dose, superovulation round and the FSH dose by round interaction were the sources of variation. If significant effects were observed in the ANOVA, differences between means were evaluated using Fisher's least significant differences procedures. Differences in the percentages of embryos developing to a particular cell stage due to FSH dose and superovulation round were determined using Chi-square procedures. All analyses were performed using the NCSS statistical software program.

Cost Analysis

For both experiments, costs of hormones needed to superovulate cows in each group were determined and divided by the number of transferable embryos recovered to calculate a cost per transferable embryo. Included in the cost analysis were cows from which no transferable embryos were collected.

Results

Experiment 1: Mean numbers of ova collected were not different ($P > 0.10$) for all cows treated with 200 vs. 400 mg FSH (4.8 $\pm$ 2.2 vs 9.0 $\pm$ 1.9, respectively), or cows treated with 100 or 200 μg GnRH (6.6 $\pm$ 1.9 vs 7.2 $\pm$ 2.1, respectively, Figure 2).The FSH x GnRH interaction was not significant. Of the 24 cows superovulated, ova were only collected from 20 cows (n = 4, 4, 7, and 5 for cows superovulated with 200 mg FSH and 100 μg GnRH, 200 mg FSH and 200 μg GnRH, 400 mg FSH and 100 μg GnRH and 400 mg FSH and 200 μg GnRH, respectively). When data from only cows in which ova were recovered (n = 20) were analyzed, mean numbers of ova were also not different ($P >$

0.10) for cows treated with 200 vs 400 mg FSH (6.0± 2.4 vs. 10.5 ± 2.0, respectively) and, similarly for cows treated with 100 or 200 μg GnRH (7.8 ± 2.0 vs. 8.8 ± 2.3, respectively, Figure 3). The FSH x GnRH interaction was not significant. Mean numbers of embryos recovered were not different (P > 0.10) between cows treated with 200 vs 400 mg FSH (4.5 ± 1.8 vs. 7.5 ± 1.5, respectively) and no difference was observed (P > 0.10) between cows treated with 100 vs 200 μg GnRH (7.2 ± 1.5 vs. 4.8 ± 1.7, respectively) (Figure 4).

The FSH x GnRH interaction was not significant. Mean number of transferable embryos was not different (P > 0.10) between cows treated with 200 vs 400 mg FSH (3.8 ± 1.3 vs. 5.3 ± 1.1, respectively), and, likewise, no difference was observed (P > 0.10) between cows treated with 100 vs 200 μg GnRH (5.6 ± 1.1 vs 3.5 ± 1.2, respectively) (Figure 5). The FSH x GnRH interaction was not significant.

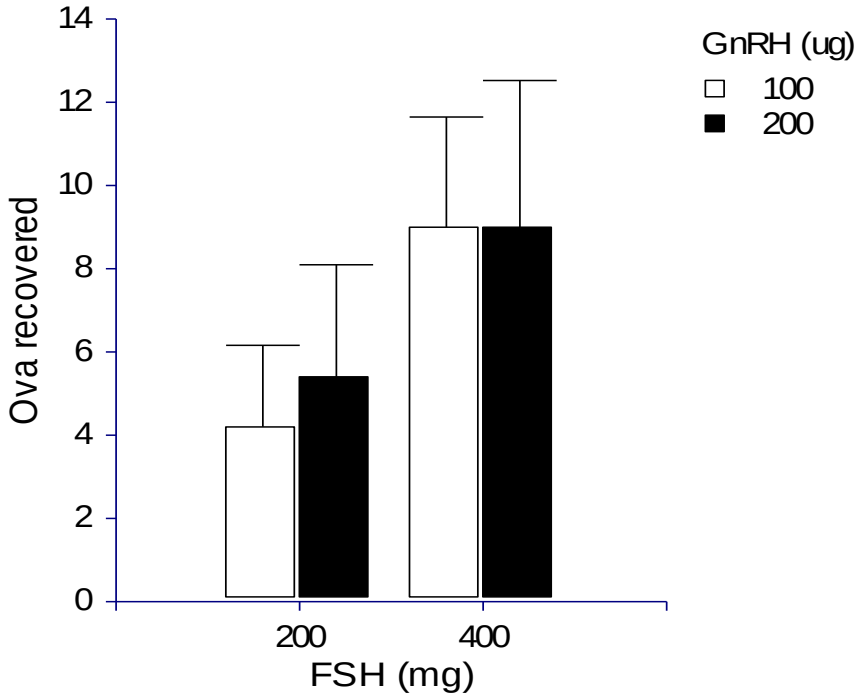

Figure 2. Mean numbers of ova recovered from all cows superovulated with two doses of FSH and GnRH.

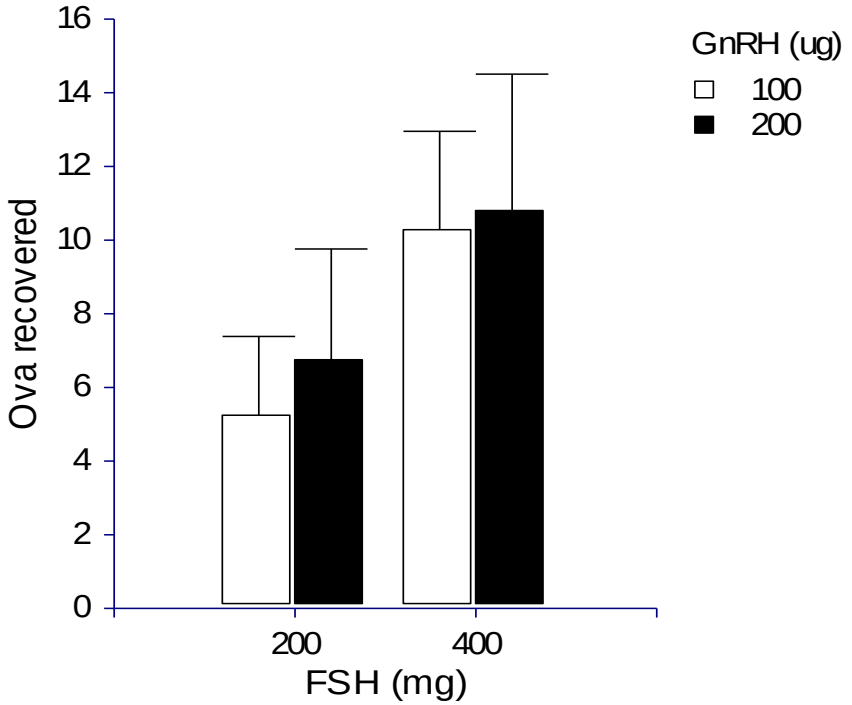

Figure 3. Mean numbers of ova recovered from cows superovulated with two doses of FSH and GnRH, in which at least one ovum was collected.

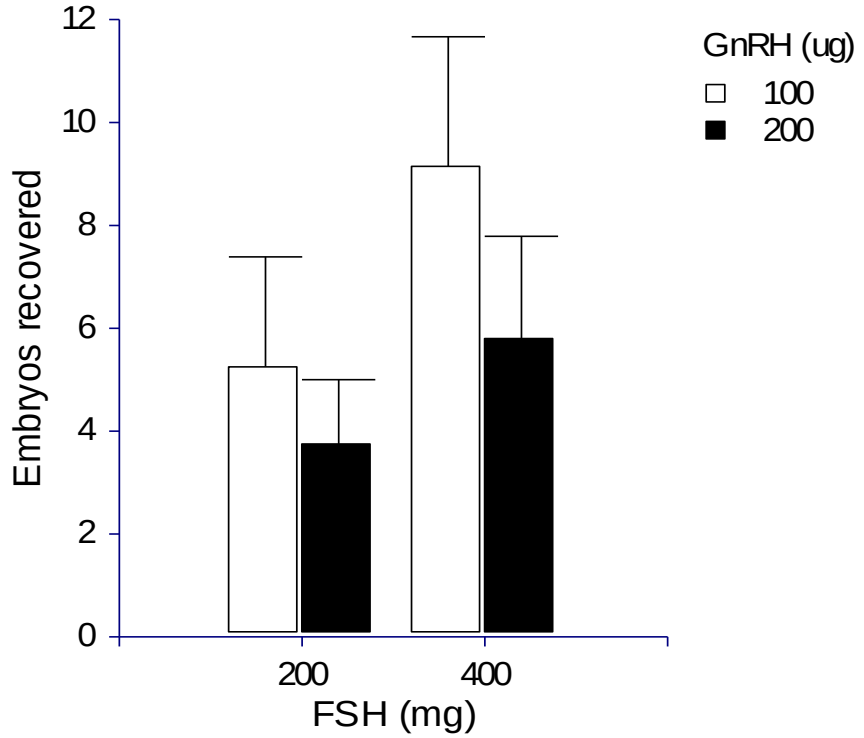

Figure 4. Mean numbers of embryos recovered from cows superovulated with two doses of FSH and GnRH, in which at least one ovum was collected.

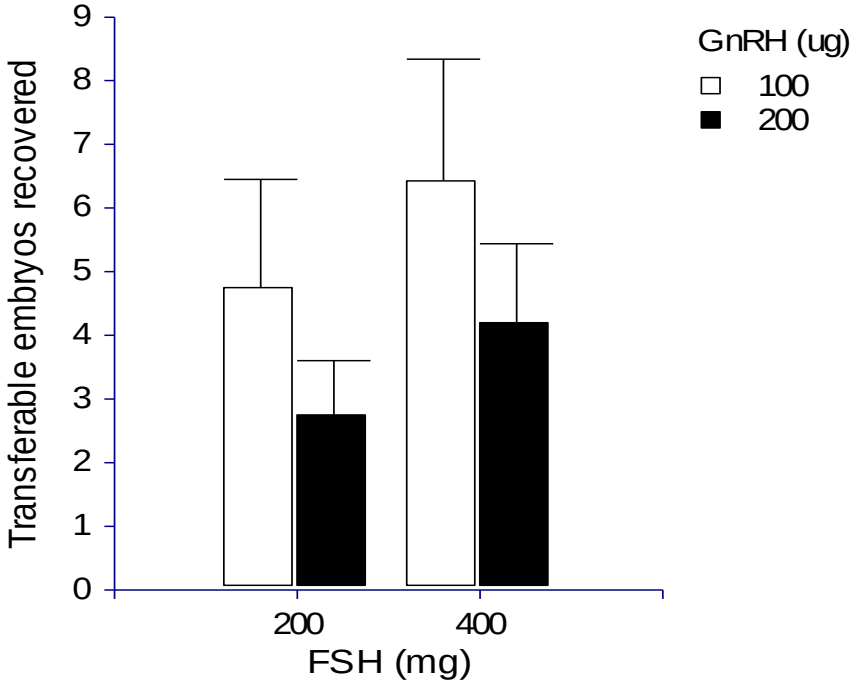

Figure 5. Mean numbers of transferable embryos collected from cows superovulated with two doses of FSH and GnRH in which at least one ovum was collected.

Mean numbers of degenerate embryos were not different ($P > 0.10$) between cows treated with 200 vs 400 mg FSH (0.8 $\pm$ 0.7 vs 2.2 $\pm$ 0.6, respectively), and no difference was observed ($P > 0.10$) between cows treated with 100 or 200 μg GnRH (1.6 $\pm$ 0.6 vs 1.3 $\pm$ 0.7, respectively, Figure 6).The FSH x GnRH interaction was not significant. Mean numbers of unfertilized ova were not different ($P > 0.10$) between cows treated with 200 vs 400 mg FSH (1.5 $\pm$ 1.0 vs 3.1 $\pm$ 0.8, respectively), however a difference ($P = 0.02$) was observed between cows treated with 100 or 200 μg GnRH (0.6 $\pm$ 0.9 vs 4.0 $\pm$ 1.0, respectively, Figure 7). The FSH x GnRH interaction was not significant.

Mean percentages of embryos recovered were not different ($P > 0.10$) for cows treated with 200 vs 400 mg FSH (84.8 $\pm$ 10.2 vs 72.8 $\pm$ 8.3, respectively), and no difference ($P > 0.10$) was observed for cows treated with 100 or 200 μg GnRH (89.0 $\pm$ 8.7 vs 68.6 $\pm$9.6, respectively, Table 1). The FSH x GnRH interaction was not significant. Mean percentage of transferable embryos recovered was greater ($P = 0.07$) for cows treated with 200 vs 400 mg FSH (77.3 $\pm$ 9.8 vs 52.9 $\pm$ 8.0, respectively), however, mean percentages of transferable embryos did not differ ($P > 0.10$) for cows treated with 100 or 200 μg GnRH (75.5 $\pm$ 8.4 vs 54.8 $\pm$ 9.3, respectively). The FSH x GnRH interaction was not significant (Table 1).

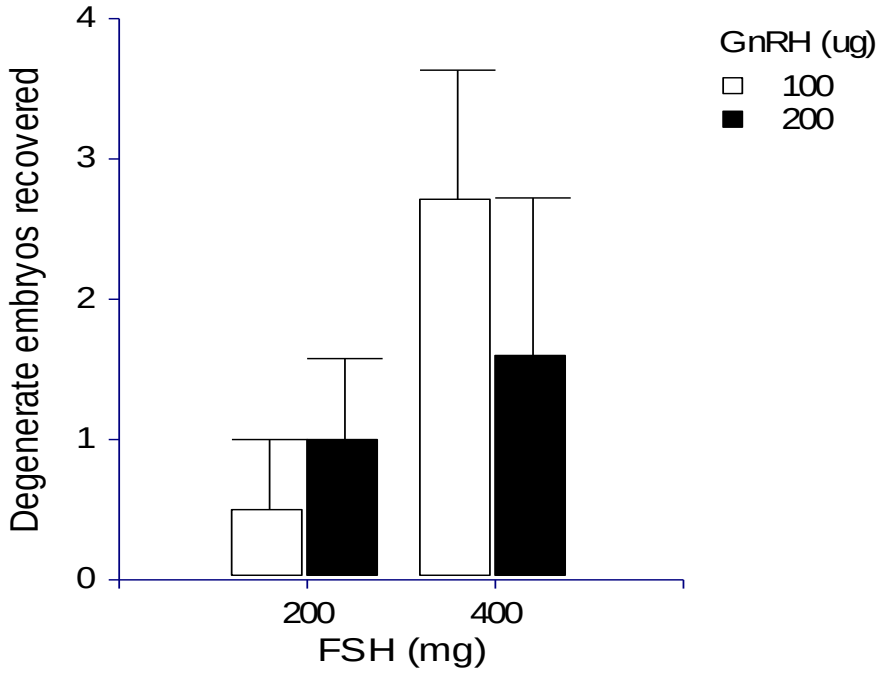

Figure 6. Mean numbers of degenerate embryos recovered from cows superovulated with two doses of FSH and GnRH in which at least one ovum was collected.

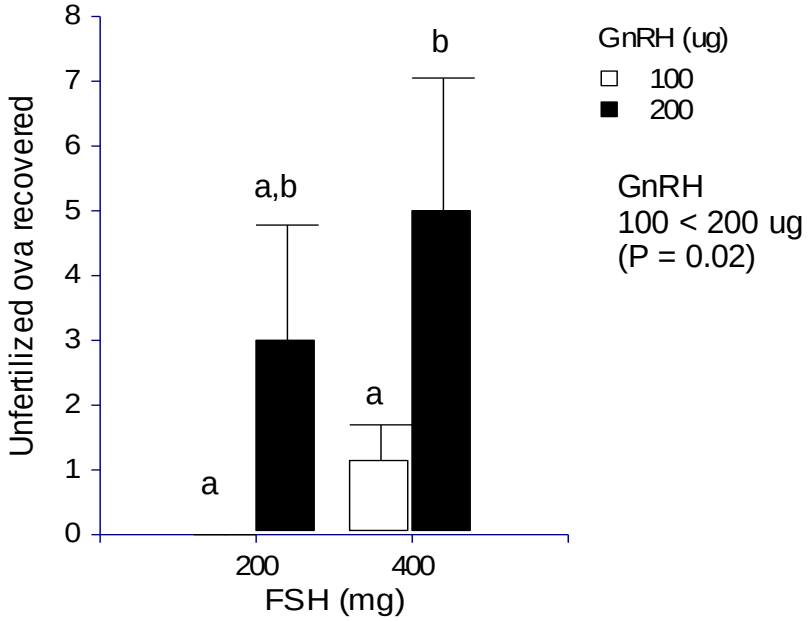

Figure 7. Mean numbers of unfertilized ova recovered from cows superovulated with two doses of FSH and GnRH in which at least one ovum was collected.

Table 1. Percentages of embryos, transferable embryos, degenerate embryos and unfertilized ova of the total ova collected from cows superovulated with 200 or 400 mg FSH and 100 or 200 µg GnRH.

Dose				**Percent**		
FSH	GnRH	n[a]	Total Embryos	Transferable Embryos	Degenerate Embryos	Unfertilized Ova
200	100	4	100 ± 0	95 ± 4[b]	4 ± 4	0 ± 0
200	200	4	69.7± 11.7	59 ± 17[b,c]	10 ± 7	30 ± 12
400	100	7	78.1 ± 13.5	56 ± 11[c]	22 ± 5	22 ± 13
400	200	5	67.4 ± 14.3	50 ± 13[c]	17 ± 10	32 ± 14

[a]Includes only cows in which ova were recovered.
[b, c] Means with different superscripts differ ($P < 0.05$).

Mean percentage of degenerate embryos was less (P = 0.10) for cows treated with 200 vs 400 mg FSH (7.5 ± 5.5 vs 19.8 ± 4.5, respectively), but no difference (P > 0.10) was observed for cows treated with 100 or 200 μg GnRH (13.5 ± 4.7 vs 13.8 ± 5.2, respectively, Table 1). The FSH x GnRH interaction was not significant. Mean percentage of unfertilized ova were not different (P > 0.10) for cows treated with 200 vs 400 mg FSH (15.1 ± 10.2 vs 27.2 ± 8.3, respectively), and, similarly, there was no difference (P > 0.10) for cows treated with 100 or 200 μg GnRH (11.0 ± 8.7 vs 31.4 ± 9.6, respectively, Table 1). The FSH x GnRH interaction was not significant.

Reduced FSH dosing yielded fewer total embryos (1.8 embryos) but a greater percentage of transferable embryos at a reduced (2/3) cost (Table 2). Increased GnRH dosing had a negative effect on the number oftransferable embryos recovered and the reduced FSH and increased GnRH dosing was not cost effective (Table 2).

Table 2. Mean numbers of transferable embryos and costs per embryo recovered ($) from donor cows superovulated with 200 or 400 mg FSH and 100 or 200 µg GnRH.

		GnRH dose (µg)		
FSH dose (mg)	n[a]	100	n	200
200	5	3.8 ± 1.6 ($24)	5	2.2 ± 0.9 ($43)
400	8	5.6 ± 1.8 ($31)	6	3.5 ± 1.2 ($50)

[a]n = total number of cows superovulated

Experiment 2: A total of 12 cows were superovulated over two rounds (n = 6 and 6 for cows treated with 200 and 400 mg FSH, respectively). Ova were recovered from four cows treated with 200 or 400 mg FSH in the first round, respectively, and four and five cows treated with 200 or 400 mg FSH in the second round, respectively. Mean numbers of ova collected were not different (P > 0.10) for cows treated with 200 vs 400 mg FSH (7.0± 2.5 vs 7.5± 2.1, respectively), and there was no difference (P > 0.10) between the first and second rounds of superovulation (7.9±1.1 vs 6.6± 1.1, respectively, Figure 8). The FSH x round interaction was not significant. Mean numbers of ova collected from cows in which at least one ovum was recovered (n = 10 and 9 in the first and second rounds of superovulation, respectively) did not differ (P< 0.10) due to FSH dose or superovulation round (Figure 9).The FSH x round interaction was not significant. Mean numbers of embryos recovered were not different (P > 0.10) for cows treated with 200 or 400 mg FSH (6.8 ± 2.5 vs 6.3 ± 2.2, respectively) and there was no difference (P > 0.10) between the first and second rounds of superovulation (7.0 ± 1.0 vs 6.0 ± 1.0, respectively, Figure 10). The FSH x round interaction was not significant. Mean numbers of transferable embryos were not different (P > 0.10) for cows treated with 200 or 400 mg FSH (5.4 ± 1.9 vs 4.3 ± 1.6, respectively) and there was no difference (P > 0.10) between the first and second rounds of superovulation (5.1 ± 0.6 vs 4.6 ± 0.6, respectively, Figure 11).The FSH x round interaction was not significant. Mean numbers of degenerate ova were not different (P > 0.10) between cows treated with 200 vs 400 mg FSH (1.4 ± 0.8 vs 2.0 ± 0.7, respectively) and there was no difference (P > 0.10) between the first and second rounds of superovulation (2.0 ± 0.5 vs 1.4 ± 0.5, respectively, Figure 12). The FSH x round interaction was not significant. Mean number of unfertilized ova

was less ($P = 0.08$) for cows treated with 200 vs 400 mg FSH (0.2 ± 0.3 vs 1.2 ± 0.3, respectively) however there was no difference ($P > 0.10$) between the first and second rounds of superovulation (0.9 ± 0.5 vs 0.5 ± 0.6, respectively, Figure 13). The FSH x round interaction was not significant.

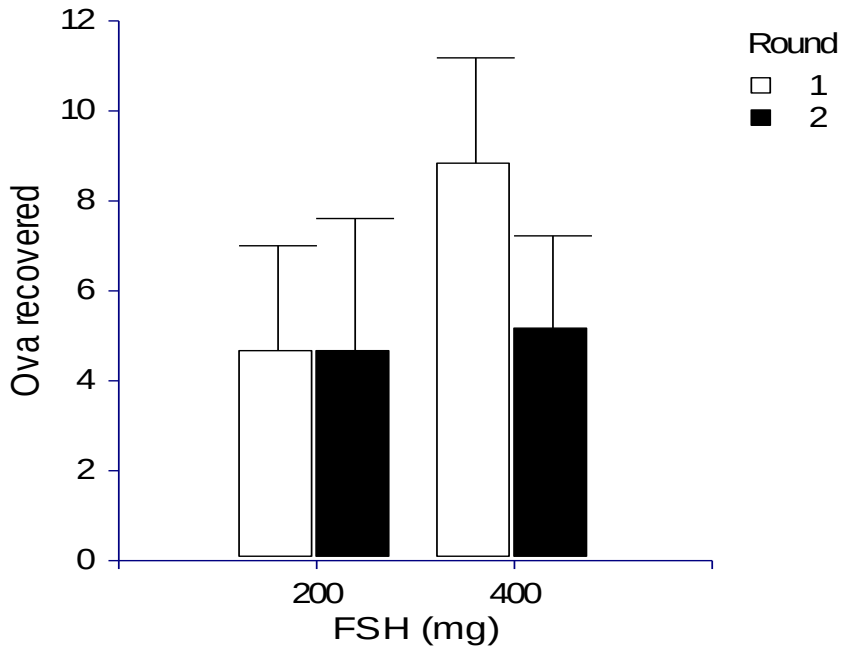

Figure 8. Mean numbers of ova recovered from all cows superovulated twice with two doses of FSH and GnRH.

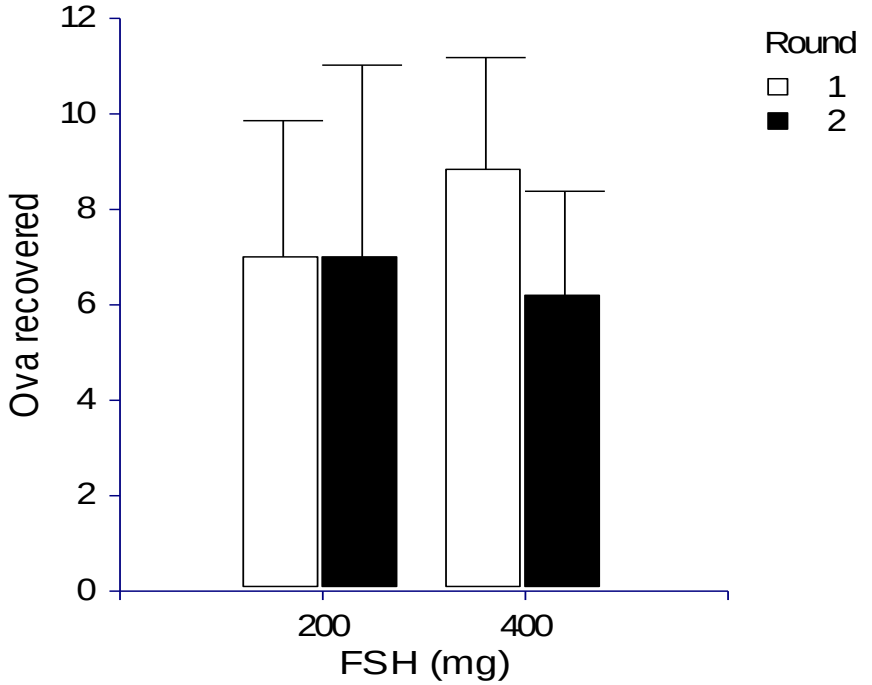

Figure 9. Mean numbers of ova collected from cows superovulated twice with two doses of FSH in which at least one ovum was collected.

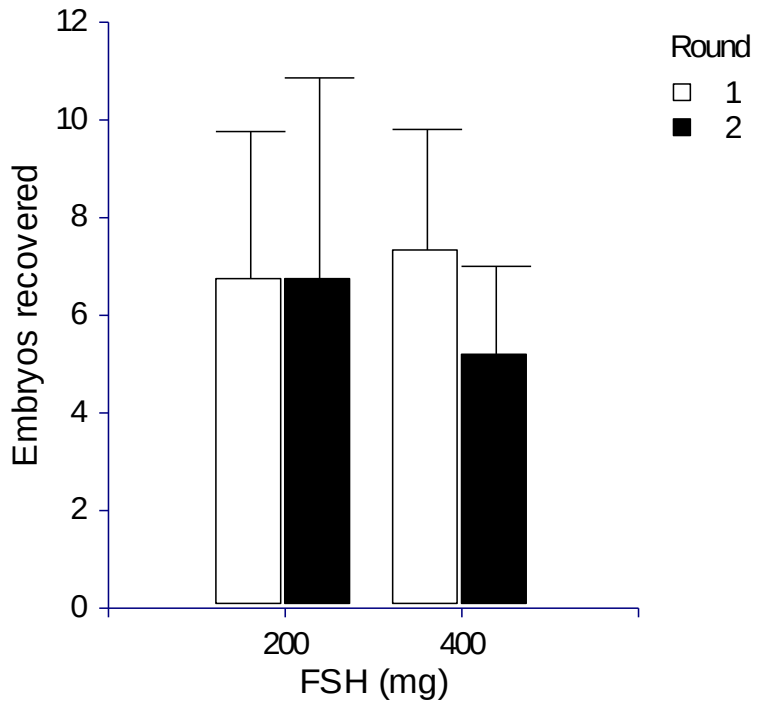

Figure 10. Mean numbers of embryos collected from cows superovulated twice with two doses of FSH in which at least one ovum was collected.

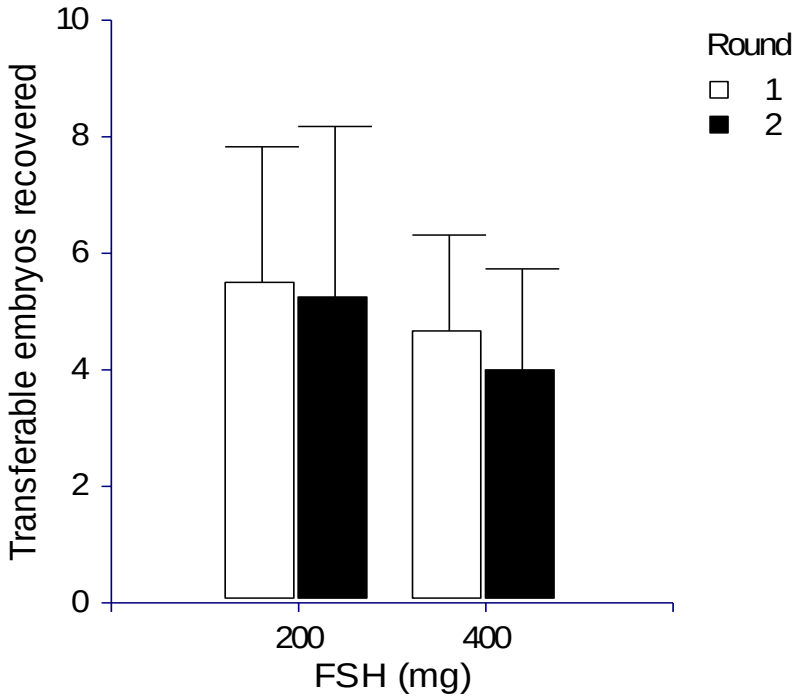

Figure 11. Mean numbers of transferable embryos collected from cows superovulated twice with two doses of FSH in which at least one ovum was collected.

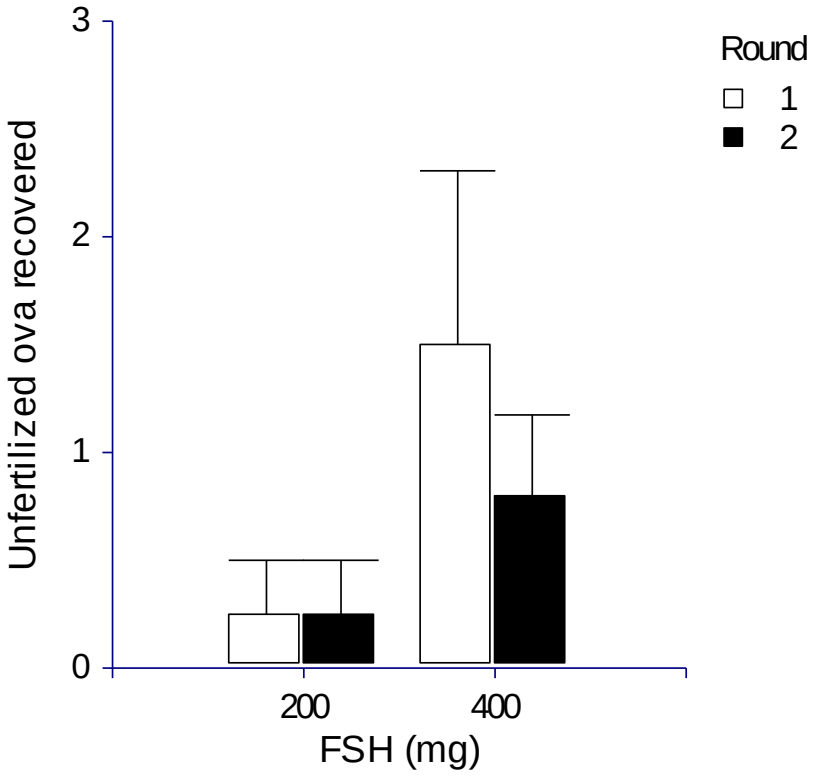

Figure 12. Mean numbers of degenerate embryos collected from cows superovulated twice with two doses of FSH in which at least one ovum was collected.

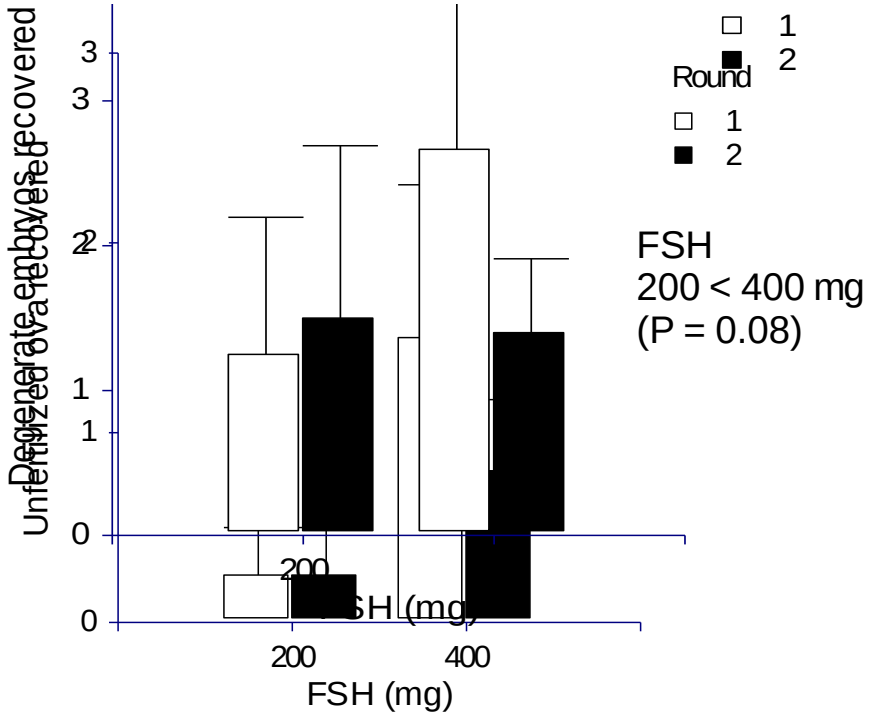

Figure 13. Mean numbers of unfertilized ova collected from cows superovulated twice with two doses of FSH in which at least one ovum was collected.

Table 3. Percentages of embryos, transferable embryos, degenerate embryos, and unfertilized ova collected from cows superovulated twice with 200 or 400 mg FSH.

Dose			Percent			
FSH	Round	n[a]	Total Embryos	Transferable Embryos	Degenerate Embryos	Unfertilized Ova
200	1	4	88 ± 12	68 ± 24	19 ± 12	12 ± 12[b]
200	2	4	92 ± 3	79 ± 7	13 ± 7	8 ± 8[c]
400	1	6	79 ± 11	55 ±12	24 ± 7	21 ± 11[d]
400	2	5	89 ± 5	50 ± 15	42 ± 17	8 ± 3[e]

[a]Includes only cows in which ova were recovered.

Mean percentages of embryos recovered were not different (P > 0.10) for cows treated with 200 or 400 mg FSH (89.6± 8.1 vs. 84.0 ± 6.9, respectively), and there was no difference (P > 0.10) between the first and second rounds of superovulation (83.3 ± 6.3 vs. 90.2 ± 6.6, respectively, Table 3). The FSH x round interaction was not significant.Mean percentages of transferable embryos was not different (P > 0.10) for cows treated with 200 or 400 mg FSH (73.6 ± 13.5 vs. 52.7± 11.5, respectively), and there was no difference (P > 0.10) between the first and second rounds of superovulation (61.9 ± 5.8 vs. 64.4 ± 6.1, respectively, Table 3). The FSH x round interaction was not significant. Mean percentages of degenerate embryos were not different (P > 0.10) between cows treated with 200 vs 400 mg FSH (16.0 ±7.7 vs. 32.7 ± 6.5, respectively), and there was no difference (P > 0.10) between the first and second rounds of superovulation (21.5 ± 9.9 vs. 27.2 ± 10.5, respectively, Table 3). The FSH x round interaction was not significant. Mean percentages of unfertilized ova were not different (P > 0.10) between cows treated with 200 vs 400 mg FSH (10.4 ± 8.1 vs. 14.6 ± 6.9, respectively), and there was no difference (P > 0.10) between the first and second rounds of superovulation (16.7 ± 5.8 vs. 8.4± 6.1, respectively, Table 3). The FSH x round interaction was not significant.

Percentage of embryos developing to the expanded and intiated hatching blastocyst stages did not differ (P > 0.10) due to FSH dose or round of superovulation (Figure 14). However, more (P = 0.04) embryos recovered from cows superovulated with 200 mg FSH completed hatching compared to 400 mg FSH. Superovulation round did not affect (P > 0.10) hatching, although the lowest incidence of hatching was observed

by embryos collected from cows superovulated with 400 mg FSH during the second round (Figure 14).

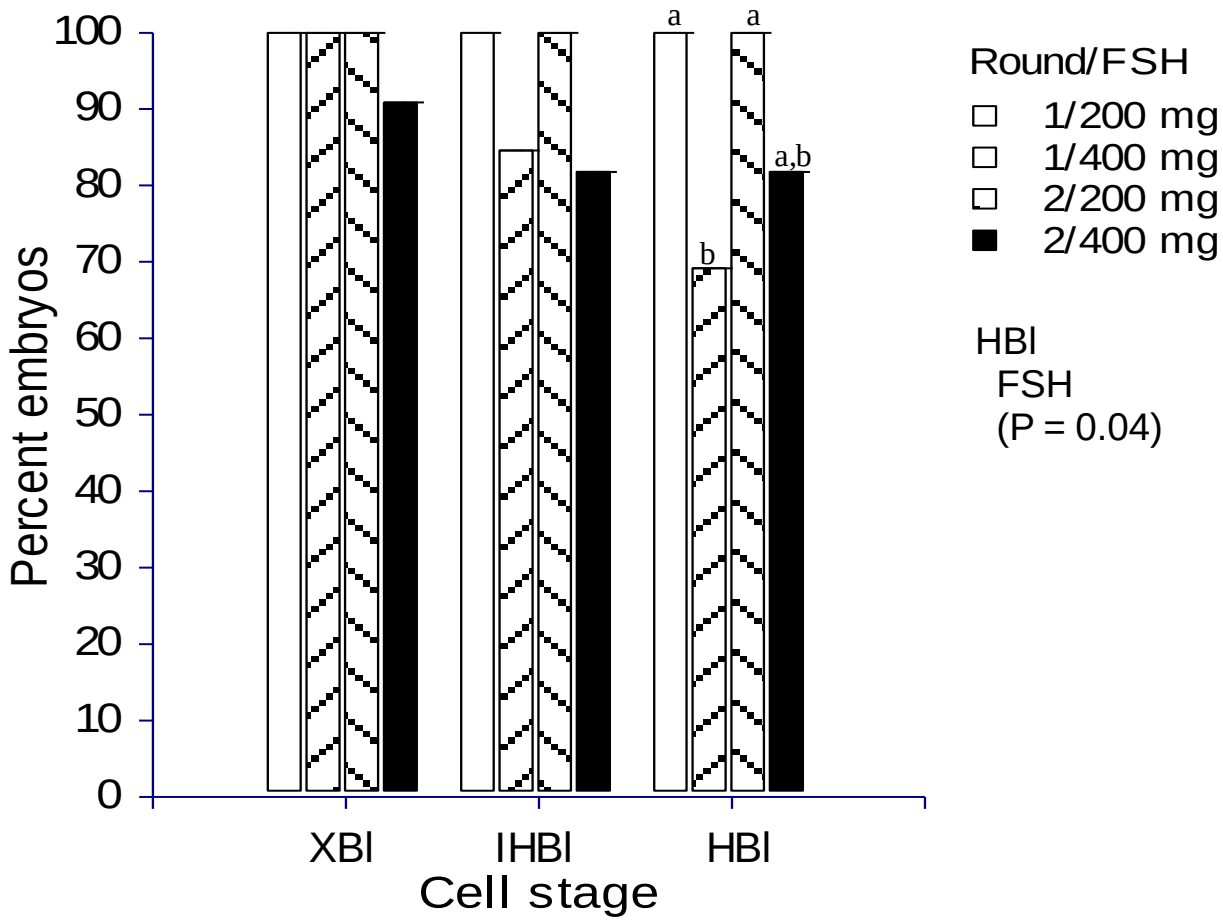

Figure 14. Percent embryos developing to the expanded (XBl), intiated hatching (IHBl) and hatched blastocyst (HBl) stages collected from cows superovulated twice with 200 or 400 mg FSH.

[a,b] Percents with different letters differ ($P < 0.05$)

Embryos collected from cows superovulated with 200 mg FSH developed to the expanded blastocyst stage sooner ($P = 0.08$) compared to cows superovulated with 400 mg FSH (28.6 $\pm$ 4.1 vs. 40.2 $\pm$ 4.0 h, respectively) regardless of round ($P > 0.10$) (35.2 $\pm$ 5.2 vs. 33.6 $\pm$ 5.8 h, respectively, Figure 15). The FSH x round interaction was not significant. Neither FSH dose (63.8 $\pm$ 7.1 vs. 59.2 $\pm$ 7.5 h for 200 vs 400 mg FSH, respectively) nor round of superovulation (62.8 $\pm$ 2.8 vs. 60.1 $\pm$ 3.1 h for round 1 vs 2, respectively) affected ($P > 0.10$) the rate of development to the initiating hatching blastocyst stage, however the FSH x round interactions are significant (Figure 15). Embryos collected from cows superovulated with 400 mg FSH in the second round initiated hatching sooner compared to embryos recovered from cows superovulated in the first round with 400 mg FSH and cows superovulated in the second round with 200 mg FSH (Figure 15).

No differences ($P > 0.10$) were observed in the rate of development to the hatched blastocyst stage due to FSH dose (82.0 $\pm$ 2.0 vs. 78.7 $\pm$ 2.2 h for 200 vs 400 mg FSH,) or superovulation round (84.0 $\pm$ 5.7 vs. 76.7 $\pm$ 6.0 h for round 1 vs. round 2, respectively). The FSH x round interaction was also not significant (Figure 15).

No difference ($P > 0.10$) was observed in PA production by embryos recovered from cows superovulated with 200 or 400 mg FSH (0.056 $\pm$ 0.026 vs. 0.038 $\pm$ 0.025 IU/mL, respectively, Figure 16). However, embryos collected from cows superovulated during round 1 produced more ($P < 0.01$) PA than embryos collected during round 2 (0.065 $\pm$ 0.009 vs. 0.028 $\pm$ 0.009 IU/mL, respectively, Figure 16). The FSH x round interaction was significant and embryos recovered from cows superovulated with 200 mg FSH in round 1 produced the greatest PA.

Similar to Experiment 1, reduced FSH dosing yielded fewer total embryos, but a greater percentage of transferable embryos at a reduced cost (Table 4). Additionally, cost per embryo increased when cows were superovulated a second time (Table 4).

Table 4. Mean numbers of transferable embryos (± SE) and costs per embryo ($) recovered from donor cows superovulated with 200 or 400 mg FSH.

		FSH dose (mg)		
Round	n*	200	n	400
1	6/4	3.7 ± 1.9 ($25)	6/4	4.7 ± 1.6 ($37)
2	6/6	3.5 ± 2.2 ($26)	6/5	3.3 ± 1.6 ($52)

*n = number of embryo collections

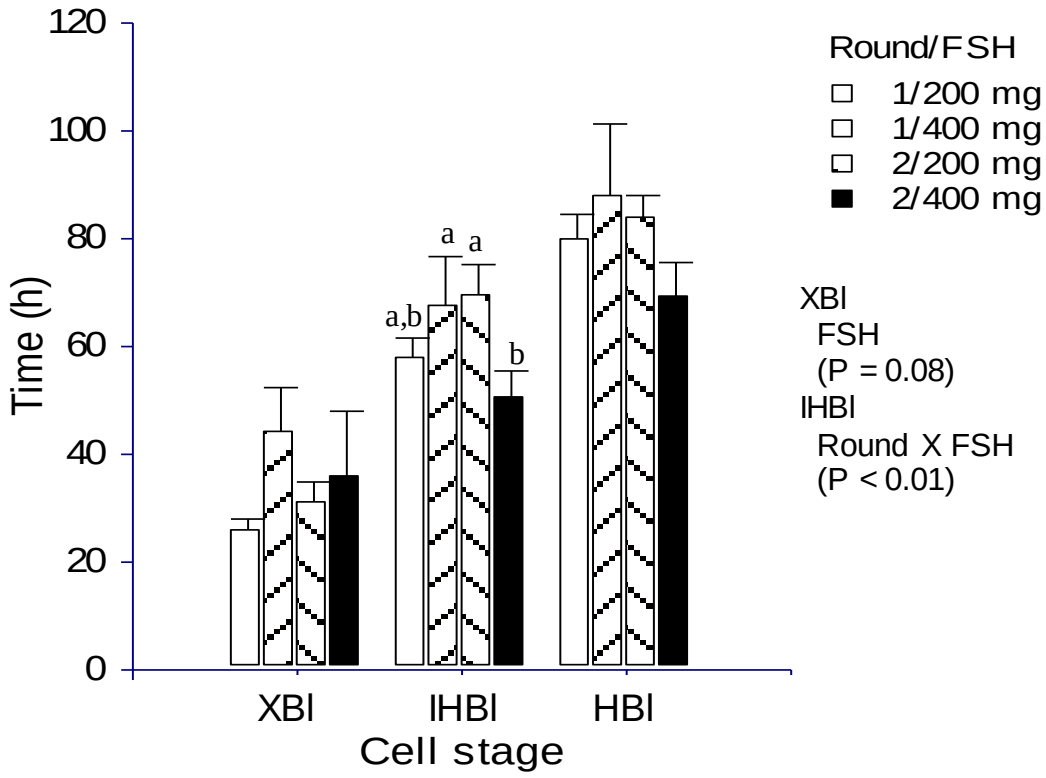

Figure 15. Times (h) required for embryos collected from cows superovulated twice with 200 or 400 mg FSH on two separate occasions to develop to the expanded (XBl), initiated hatching (IHBl) and hatched (HBl) stages.
[a, b] Means with different letters differ (P < 0.05).

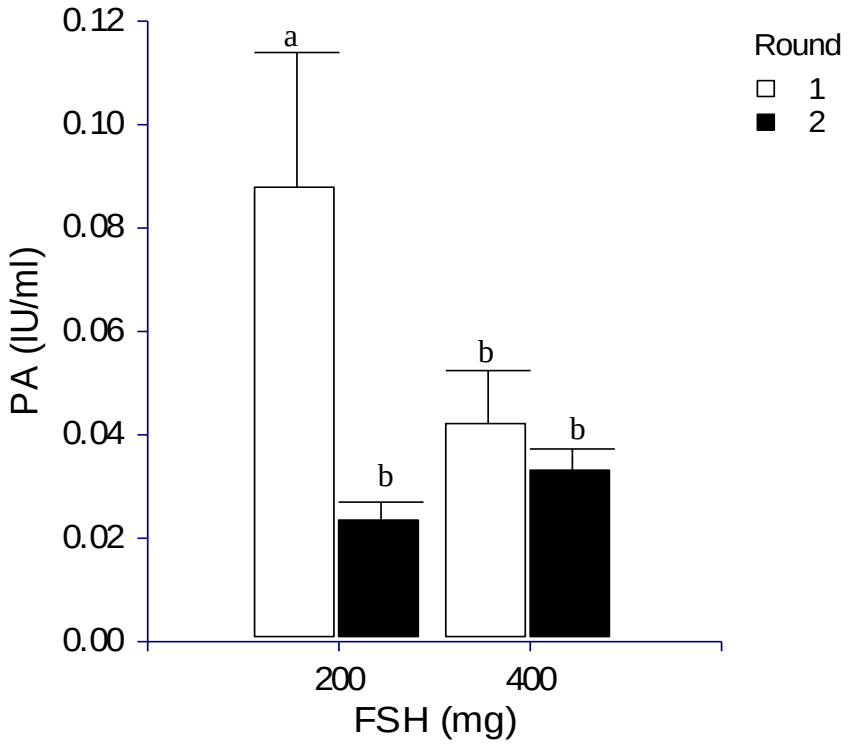

Figure 16. Plasminogen activator (PA) production (IU/mL) produced by embryos collected from cows superovulated twice with 200 or 400 mg FSH.
[a, b] Means with different letters differ ($P < 0.05$).

Discussion

Currently, superovulation is expensive and its results are variable. To date, the lowest dose of FSH necessary to exhibit a satisfactory ovarian response is not known (Gomes et al., 2014), although Lewis suggests the dose of FSH for Bos taurus cattle should be 360-400 mg (Barati et al., 2006)), and Baruselli et al. (2006) has shown that Bos indicus donors can be superovulated using less FSH than what is needed for Bos taurus donors. These two experiments, suggest that as little as 200 mg FSH is sufficient to obtain a satisfactory number of transferable embryos from Bos taurus donors. Reducing the amount of FSH given to a cow during a superovulation protocol would substantially lower the cost of the protocol.

The variability of superovulatory response may be due to FSH overdosing causing follicles beginning to undergo atresia to ovulate oocytes of inferior quality as compared to oocytes from follicles which have not begun the process of atresia, resulting in more degenerate embryos and unfertilized ova. Follicles undergoing atresia have fewer FSH receptors than those which continue to grow, but the addition of a large quantity of exogenous FSH allows the fewer number of receptors on dying follicles to bind FSH and continue to grow. It is known that superovulation causes the ovulation of smaller follicles than those which ovulate during a normal estrous cycle.

The variability of superovulation could also be due to the number of times a donor is superovulated. As the number of superovulations increases, the ovaries may become refractory to FSH, and studies have shown that exposure to the hormones used in a superovulation protocol cause a cow to build up resistance to them (Blondin et al., 1996). Kimura et al. (2007) superovulated the same group of cows every 2-3 mos for a year. The

average numbers of total ova collected from each cow during the separate rounds of superovulation was 12.0, 12.6, 6.8, 7.6, and 11.4, respectively, and the average numbers of transferable embryos collected per cow were 11.4, 10.4, 6.6, 4.8, and 10.4, respectively. Additionally, aged donor cows tend not to produce as many follicles or ovulate as many oocytes during superovulation as their younger, less superovulated counterparts (Hesser et al., 2011). As both experiments of this project used only cows which had never been superovulated prior to treatment, another aspect of Experiment 2 was to superovulate the cows a second time. No differences were observed in any of the endpoints which were attributable to how many times a cow had been superovulated. The variability associated with superovulation could also be due to weather or the donor's age, diet and environment.

In Experiment 1, reduced Folltropin V dosing yielded fewer total embryos, but a greater percentage of transferable embryos than the other dosing strategies. This could be due to the lower dose of FSH not rescuing as many follicles from atresia as the higher dose, as there was not a high enough concentration of FSH to bind follicles with few FSH receptors, so follicles undergoing atresia did not ovulate an oocyte, which means the overall quality of the oocytes ovulated was improved. In Experiment 2, cows receiving 200 mg Folltropin V yielded fewer total embryos but a greater percentage of transferable embryos and fewer unfertilized ova as than cows receiving 400 mg Folltropin V during the course of a superovulation protocol.

A second hormone given during a superovulation protocol, GnRH, is administered when the donor animals first exhibit heat. This is done to increase the number of ova ovulated, but a benefit of injecting cows with GnRH has not yet been

realized with respect to improving overall embryo production. This could be due to underdosing donors with GnRH, and, in Experiment 1, cows treated with 200 µg GnRH produced more unfertilized ova than cows treated with 100 µg GnRH. Similarly, Dias et al. (2010) observedcows superovulated with a higher dose of GnRH (200 µg) ovulated higher numbers of ova when superovulated as compared to cows being superovulated with 100 µg GnRH. Although more ova were recovered, the number of unfertilized ova recovered increased with the higher dose.

The most cost effective treatment from Experiment 1 was 200 mg FSH and 100 µg GnRH. The high GnRH dosing strategy was found not to be cost effective, so Experiment 2 focused on the differences between the two FSH doses only. Experiment 2 also showed that cows superovulated with 200 mg FSH produced fewer unfertilized ova than cows superovulated with 400 mg FSH. The most cost effective treatment from Experiment 1 was 200 mg FSH during the first round of superovulation.

Hatching is an important step of embryo development, and plasminogen activator is one enzyme that has been shown to aid in hatching. Mendoza et al. (2012) found that embryos producing at least 0.3 mIU/mL/h are more likely to generate pregnancies following transfer. In Experiment 2, embryos collected from cows superovulated with 200 mg FSH during the first round of superovulation produced more plasminogen activator than embryos in any other group.

Conclusion

Although cows superovulated with the standard doses of FSH (400 mg) and GnRH (100 µg) produced more total ova, many were either unfertilized or, if fertilized,

not transferable embryos. These data suggest that the higher dosing is likely inducing ovulation of poorer quality ova which fail to fertilize and generate competent embryos. Producers involved in embryo transfer may be served better by starting their donors on a reduced dose of FSH then adjusting to a higher dose as the superovulatory response is observed to decrease. The reduced dose not only contributes to a reduced cost but also extends the longevity of a cow to serve as a donor.

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CHAPTER 4

DEVELOPMENT OF A DIPSTICK STYLE ASSAY TO ASSESS EMBRYO VIABILITY AND PREDICT SUCCESS OF PREGNANCY ESTABLISHMENT FOLLOWING TRANSFER

Abstract

The objective of this project was to develop a dipstick-style assay to detect and quantify urokinase-type plasminogen activator (uPA) in embryo-conditioned medium (ECM) that could be used on the farm to grade embryos during an embryo transfer. The first set of experiments constructed dipsticks where 5 µL of 1, 10 or 50 mM of the uPA synthetic colorimetric tripeptide substrate glutamic acid-glycine-arginine (EGR) were pipetted and dried on strips of cellulose acetate, chromatography paper, glass fiber membrane or nitrocellulose. Dipsticks were incubated for 90 min in 96-well plates in solutions containing 25 µL 0, 1, 10, or 100 IU human urokinase (UK)/mL or ECM. Presence and intensity of yellow color arising from enzymatic cleavage of the substrate on the dipsticks were recorded using a 3-point numeric systemat 30-min intervals during the incubation period. The next set of experiments was carried out in 96-well plates containing 25 µL of 1, 5, 10, 20 or 50 mM EGR or 50 µL of 10 or 20 mM EGR and 25 µL 0, 1, 10, or 100 IU UK/mL or ECM. Presence and intensity of yellow color in the wells arising from enzymatic cleavage of the substrate were recorded as described for the strips. Optical densities at 405 nm (OD405) were also determined using an ELISA plate reader and these values were recorded and compared to the visual data. A distinct yellow color was observed on all dipsticks constructed with the four substrates after 60 min of incubation in wells containing 100 IU UK/mL and 10 or 50 mM EGR. Similarly, a

distinct yellow color was visually observed in wells containing 100 IU UK/mL and 25 µL of 1, 5, 10 or 50 mM EGR or 50 µL of 10 mM EGR after 30 min of incubation. ECM failed to generate color detected by visual observation either on the dipsticks or in the plates but scanning the plate with the plate reader detected changes in OD405 within 30 min of incubation. Although the dipstick style and the ELISA plate based assays were successful in detecting UK activity by visual observation, neither were sensitive enough to detect uPA in ECM. However, uPA activity in ECM could be quantified using an ELISA plate reader. The requirement for a plate reader may limit application of the plate assay to on farm embryo transfers.

Introduction

Although embryo transfer is a popular technique in the cattle industry, it is expensive and culturing embryos to determine viability is time consuming and not practical. Considering the expense, it is important to ensure a high proportion of embryos transfered to recipients will generate and maintain pregnancies. In an attempt to select embryos with a high probability of pregnancy establishment, multiple grading schemes have evolved to assess embryo quality. Two grading schemes are commonly used to assess quality of a bovine embryo destined for transfer. The first grades transferable embryos on a scale from 2-4, with 4 being the best quality (a grade of 1 describes an unfertilized ovum). The second grading scheme uses four ranks: poor, fair, good and excellent, to determine the likelihood of a transfered embryo generating and maintaining a pregnancy. Both of these schemes are based solely on observation of gross morphology where embryos just prior to transfer are evaluated microscopically for flaws. Results

using these grading schemes are variable; 71% of grade 4 embryos and 45% of excellent quality embryos generated pregnancies following transfer (Mendoza et al., 2011). Clearly, there is a need for a more accurate assessment of an embryo's likelihood to establish a pregnancy.

The objective of this project was to create a dipstick-style assay to measure urokinse-type plasminogen activator (uPA) production by a single bovine embryo. Urokinse-type plasminogen activator is undetectable in bovine embryos until formation of the blastocoel at which time production increases as the blastocyst expands and starts to shed the zona pellucida in the process referred to as hatching (Menino and Williams, 1987). Urokinse-type plasminogen activator production remains elevated throughout and after hatching. Kaaekuahiwi and Menino (1990) reported that uPA was postively correlated with embryonic size, developmental stage and cell number. Detection and quantification of uPA produced by an embryo using a rapid dipstick assay could a quick and reliable way used on the farm to assess which embryos would be best suited for transfer to recipients.

Materials and Methods

Dipstick Tests. Initially, dipsticks were constructed by cutting strips of nitrocellulose with dimensions of 5 mm X 25 mm and pipetting and drying 5 μL of 1 mM of the uPA synthetic colorimetric tripeptide substrate glu-gly-arg (EGR) on the end of the strips. Dipsticks were incubated in wells in a 96-well plate containing 25 μL of Ham's F-12 medium with 1.5% BSA and 0, 1, 10, or 100 IU human urokinase(UK)/mL and visually observed for color development at 30-min intervals for up to 90 min. A yellow

color was expected to appear on the dipstick proportionate to the amount of EGR cleaved by UK. Color development was scored using the following 3-point system where: 0 = absence of yellow, 1 = light yellow and 2 = bright yellow.

In subsequent experiments, dipsticks were constructed by attaching 5 X 5 mm squares of cellulose acetate, chromatography paper, glass fiber membrane or nitrocellulose onto the ends of 5 X 25 mm plastic strips. Five microliters of 1, 10 or 50mM EGR were pipetted onto the 5 X 5 mm squares and dried. Dipsticks were incubated in wells in a 96-well plate containing 25 μL of medium with 0, 1, 10, or 100 IU UK/mL or embryo-conditioned medium (ECM), visually observed and scored for color development as described at 30-min intervals for up to 90 min.

Well Tests. An alternative assay was developed with EGR using 96-well plates. Twenty-five microliters of 1, 5, 10, 20 or 50 mM EGR were combined with 25μL of medium containing 0, 1, 10, or 100 IU UK/mL or ECM in wells in a 96-well plate. In a second series of experiments 50 μL of 10 or 20 mM EGR were combined with 25μL of medium containing 0, 1, 10, or 100 IU UK/mL or ECM. Plates were covered and placed on a slide warming tray at 37°C. Wells were observed and scored for color development visually as described for the dipsticks and photometrically at OD405 using an ELISA plate reader at 30-min intervals for up to 120 min. Wells were fixed with 50 μL 50% acetic acid at the end of the incubation.

Urokinase-type plasminogen activator activity in ECM was computed from standard curves constructed from OD405 in the well assay using 25 μL 50 mM EGR and 0, 1, 10 and 100 IU UK/mL at 30, 60, 90 and 120 min of incubation. A standard curve

was also constructed using the mean OD405 computed for each standard over the 120 min incubation. For comparison, uPA activity in ECM was determined using the caseinolytic agar gel assay described by Menino and Williams (1987).

Results

Dipstick Tests. At a concentration of 1 mM EGR, visible results were present on dipsticks constructed with chromatography paper and glass fiber membrane after incubation in 1, 10 and 100 IU UK/mL and on cellulose acetate after incubation in 100 IU UK/mL for 90 min(Figure 17) (Table 5). When 10 mM EGR was pipetted onto the strip, yellow color was observed on all substrates after 60 min when incubated with 100 IU UK/mL (Table 6). Chromatography paper and nitrocellulose produced visual results after 30 min of incubation at a concentration of 100 IU UK/mL (Table 6). Yellow color was observed on all four substrates with 50 mM EGR after 60 min of incubation when strips were placed in wells containing 100 IU UK/mL (Table 7). Color development was not observed on strips with 1, 10 or 50 mM EGR following incubation for up to 90 min in ECM.

Table 5. Effects of matrix and duration of incubation on visual detection of 1 mM glu-gly-arg tripeptide (EGR) hydrolysis by urokinase (UK).

Matrix[a]	UK (IU/mL)	Incubation time (min) 0	30	60	90
CA[b]	0	0	0	0	0
CA	1	0	0	0	0
CA	10	0	0	0	0
CA	100	0	0	0	1
CP[c]	0	0	0	0	0
CP	1	0	0	0	0.5 ± 0.5
CP	10	0	0	0	1.0 ± 1.0
CP	100	0	0	0	1.0 ±1.0
GFM[d]	0	0	0	0	0
GFM	1	0	0	0	1
GFM	10	0	0	0	1
GFM	100	0	0	0	2
NC[e]	0	0	0	0	0
NC	1	0	0	0	0
NC	10	0	0	0	0
NC	100	0	0	0	0

[a]5 µl EGR was applied to each matrix
[b]CA = cellulose acetate
[c]CP = chromatography paper
[d]GFM = glass fiber membrane
[e]NC = nitrocellulose

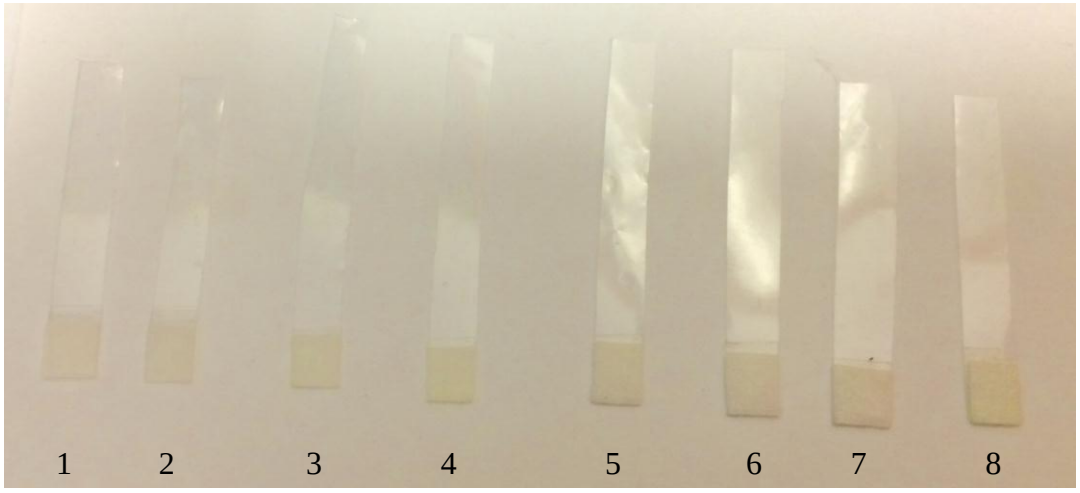

Figure 17. Dipstick assays constructed with chromatography paper (1-4) and glass fiber membrane (5-8) and 1 mM EGR after incubation in 0, 1, 10, and 100 IU urokinase/mL (left to right).

Table 6. Effects of matrix and duration of incubation on visual detection of 10 mM glu-gly-arg tripeptide (EGR) hydrolysis by urokinase (UK).

Matrix[a]	UK (IU/mL)	Incubation time (min) 0	30	60	90
CA[b]	0	0	0	0	0
CA	1	0	0	0	0
CA	10	0	0	0	0
CA	100	0	0	0.25 ± 0.25	1.25 ± 0.48
CP[c]	0	0	0	0	0
CP	1	0	0	0	0
CP	10	0	0	0	0
CP	100	0	0.25 ± 0.25	0.25 ± 0.25	0.75 ± 0.48
GFM[d]	0	0	0	0	0
GFM	1	0	0	0	0
GFM	10	0	0	0	0
GFM	100	0	0	0.5 ± 0.29	1.5 ± 0.29
NC[e]	0	0	0	0	0
NC	1	0	0	0	0
NC	10	0	0	0	0
NC	100	0	0.25 ± 0.25	0.5 ± 0.29	1.5 ± 0.29

[a]5 µl EGR was applied to each matrix
[b]CA = cellulose acetate
[c]CP = chromatography paper
[d]GFM = glass fiber membrane

Table 7. Effects of matrix and duration of incubation on visual detection of 50 mM glu-gly-arg tripeptide (EGR) hydrolysis by urokinase (UK).

Matrix[a]	UK (IU/mL)	Incubation time (min)			
		0	30	60	90
CA[b]	0	0	0	0	0
CA	1	0	0	0	0
CA	10	0	0	0	0
CA	100	0	0	2	2
CP[c]	0	0	0	0	0
CP	1	0	0	0	0
CP	10	0	0	0	2
CP	100	0	0	2	2
GFM[d]	0	0	0	0	0
GFM	1	0	0	0	0
GFM	10	0	0	0	2
GFM	100	0	0	2	2
NC[e]	0	0	0	0	0
NC	1	0	0	0	0
NC	10	0	0	0	0
NC	100	0	0	2	2

[a]5 µl EGR was applied to each matrix
[b]CA = cellulose acetate
[c]CP = chromatography paper
[d]GFM = glass fiber membrane
[e]NC = nitrocellulose

Well Tests. A faint yellow color was observed in wells containing 25 μL 1 mM EGR and 100 IU UK/mL after 30 min and progressed to bright yellow after 60 min of incubation (Figure 18A). Faint yellow color was observed in wells containing 1mM EGR and 10 IU UK/mL at 90 min of incubation (Figure 18A). Wells containing 5 mM EGR and 100 IU UK /mL showed a faint yellow color after 30 min which increased after 60 min of incubation (Figure 18B). Wells containing 0, 1 or 10 IU UK/mL did not exhibit any color development at any time point (Figure 18B). Yellow color was clearly observed after 30, 60, and 90 min of incubation in wells containing 10 mM EGR and 100 IU UK/mL (Figure 18C). Wells containing 10 mM EGR and 10 IU UK/mL showed a faint yellow color at 90 min of incubation (Figure 18C). Color development was first observed with 100 IU UK/mL after 60 min of incubation in wells containing 20 mM EGR but wells with 0, 1 or 10 IU UK/mL did not exhibit any color development at any time point (Figure 18D). Color development was observed in wells containing 50 mM EGR and 100 IU UK/mL after 30 min which continued to increase to 120 min of incubation (Figure 18E). Wells containing 0, 1 or 10 IU UK/mL or ECM did not develop detectable color at any time point (Figure 18E). When these plates were scanned with the plate reader, changes in OD405 were observed in wells containing 50 mM EGR and 100 IU UK/mL after 30 min which continued to increase in linear fashion up to 120 min of incubation (Figure 19). Slight changes in OD405 were observed in wells containing10 IU UK/mL after 30 min which also increased in linear fashion up to 120 min of incubation but with a much reduced slope. An OD405 similar to the OD405 for 100 IU UK/mL at 30 min of incubation was observed in wells containing ECM-1 which dropped at 60 min but

remained between OD405 for 10 and 100 IU UK/mL up to 120 min of incubation (Figure 19).

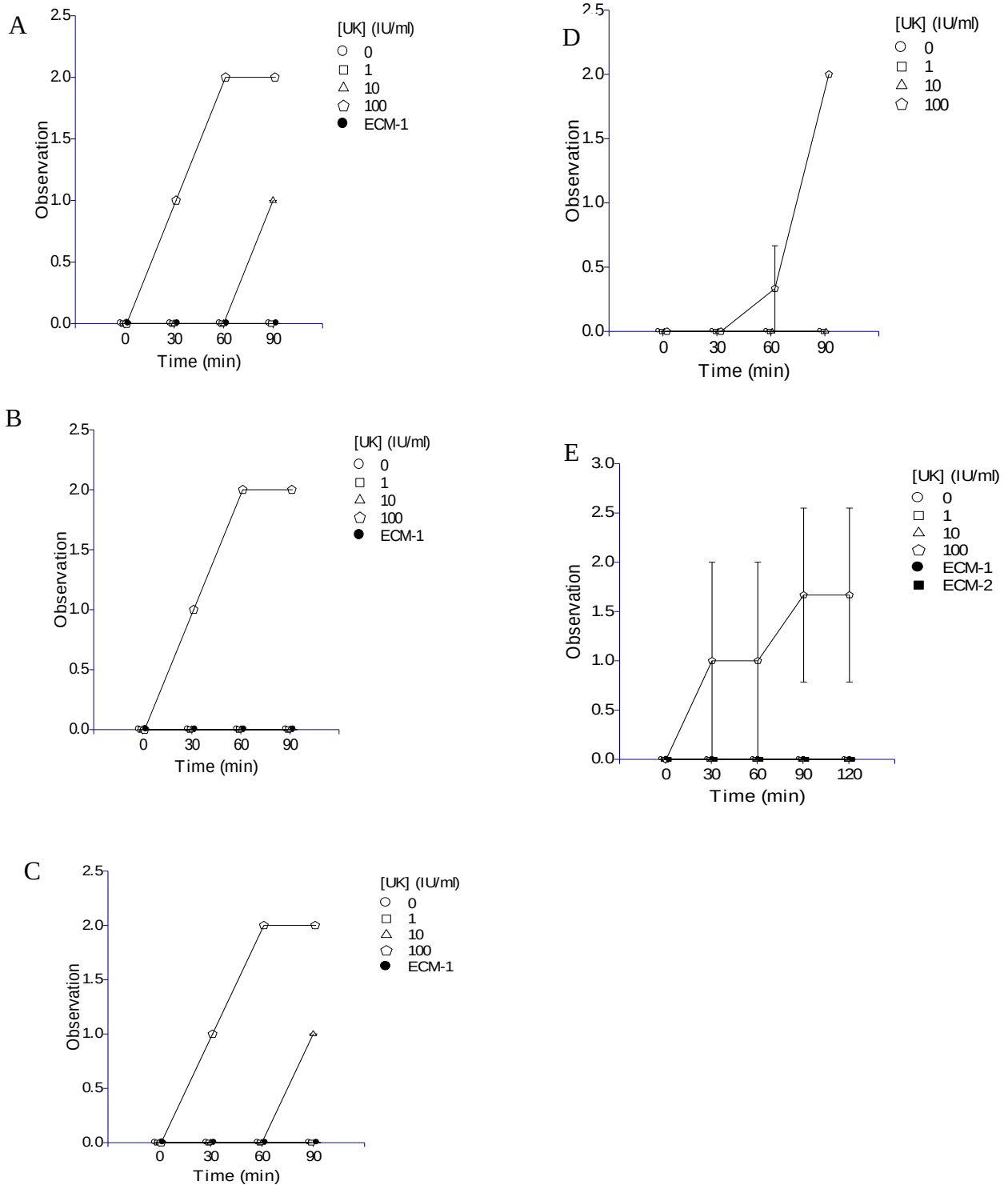

Figure 18. Visual detection of yellow color in wells containing 25 µL A) 1, B) 5, C) 10, D) 20 and E) 50 mM EGR and 25 µL 0, 1, 10 or 100 IU human urokinase (UK)/mL or embryo conditioned medium (ECM-1,-2).

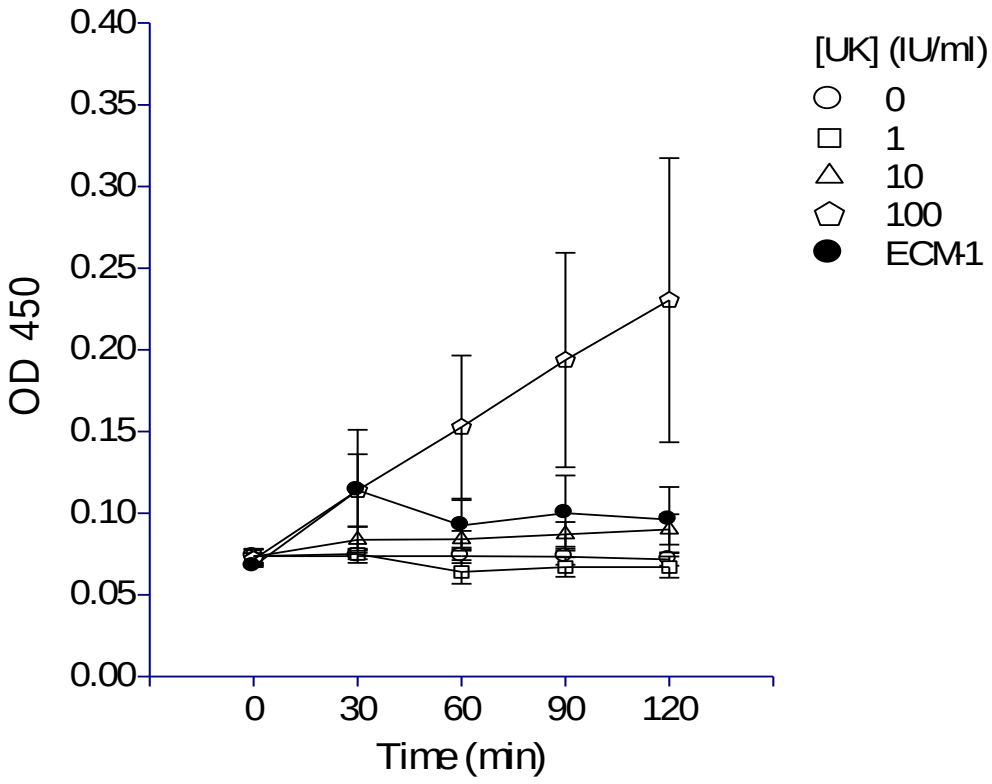

Figure 19. Changes inoptical densities at 405 nm (OD405) in wells containing 25 µL 50 mM EGR and 25 µL 0, 1, 10 or 100 IU human urokinase (UK)/mL or embryo conditioned medium (ECM-1).

A faint yellow color was observed in wells containing 50 μL 10 mM EGR and 1, 10 or 100 IU UK/mL after 30 min and throughout 90 min of incubation (Figure 20). However when the concentration was increased to 20 mM EGR, a faint yellow color was observed only in 100 IU UK/mL after 90 min which progressed to bright yellow after 120 min of incubation (Figure 21). When scanned with the plate reader, changes in OD405 were observed in wells containing 20 mM EGR and 1, 10 and 100 IU UK/mL after 30 min which for 10 and 100 IU UK/mL continued to increase up to 120 min of incubation (Figure 22).

Urokinase-type plasminogen activator activity in ECM could be determined from the well assay as early as 30 min after incubation in 25 μL 50 mM EGR (Table 8). However, as indicated by the standard error, considerable variation was associated with the activity computed from the 30-min assay. Much less variation was associated with uPA activities computed from OD405 measured at 60, 90 and 120 min and the mean OD405. Interestingly, from the OD405 vs UK graph in Figure 17 and the data in Table 8 it appears the ECM may be losing activity as the incubation progressed. Urokinase-type plasminogen activator activity determined using the EGR assay did not compare closely to activity calculated using the caseinolytic agar gel assay (Table 8).

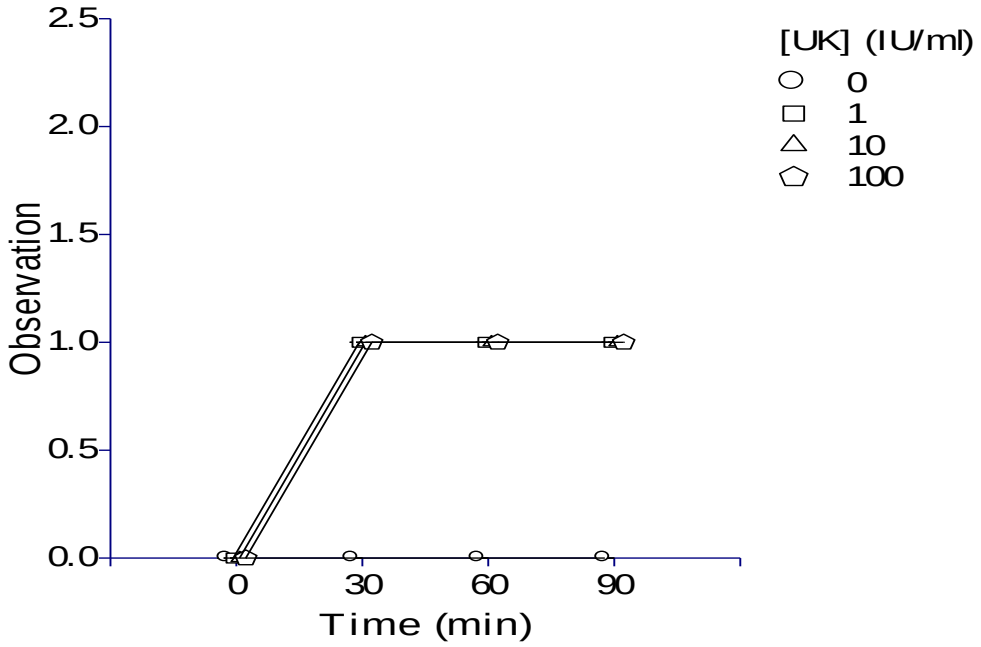

Figure 20. Visual detection of yellow color in wells containing 50 µL 10 mM EGR and 25 µL 0, 1, 10 or 100 IU human urokinase (UK)/mL.

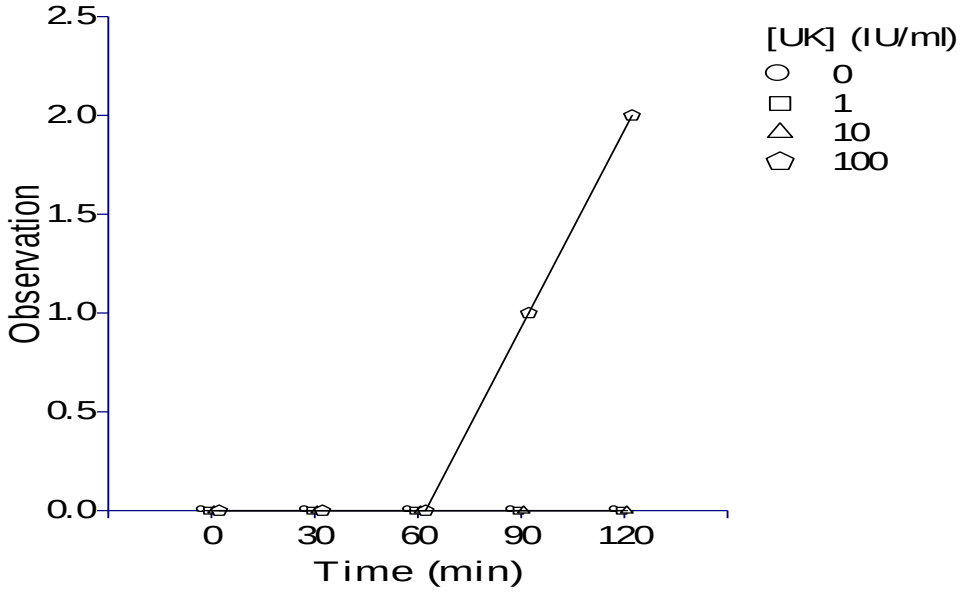

Figure 21. Visual detection of yellow color in wells containing 50 µL 20 mM EGR and 25 µL 0, 1, 10 or 100 IU human urokinase (UK)/mL.

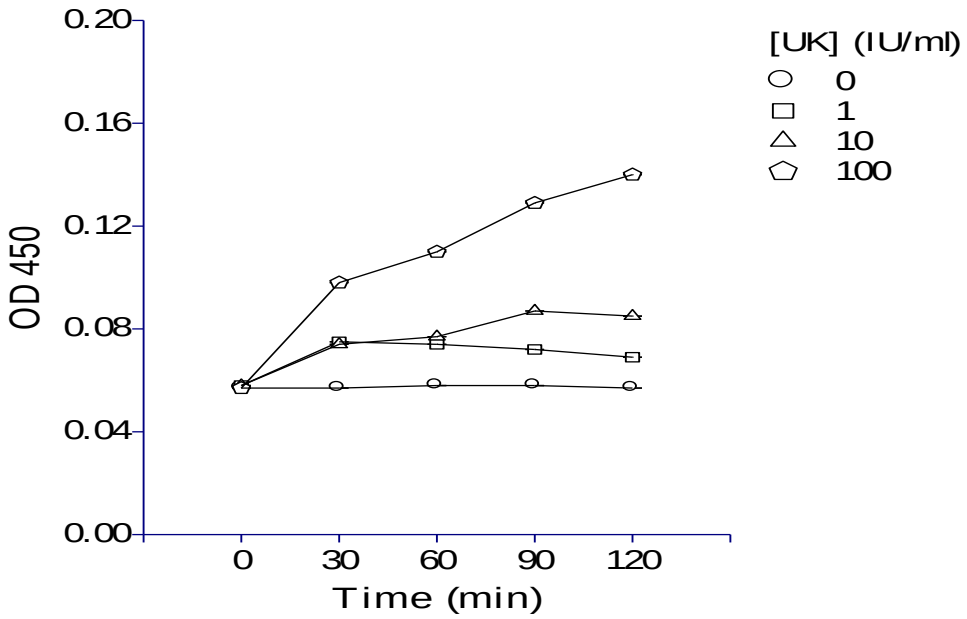

Figure 22. Changes inoptical densities at 405 nm (OD405) in wells containing 50 µL 20 mM EGR and 25 µL 0, 1, 10 or 100 IU human urokinase (UK)/mL.

Table 8. Urokinase-type plasminogen activator (uPA) concentrations (IU/ml) in embryo-conditioned medium (ECM) determined using the glu-gly-arg tripeptide (EGR) or caseinolytic agar gel assay.

Assay	ECM uPA (IU/ml)
EGR_{30}[a]	50.6 ± 41.4
EGR_{60}	17.3 ± 8.7
EGR_{90}	11.9 ± 8.8
EGR_{120}	8.3 ± 6.1
EGR_{mean}[b]	15.7 ± 11.1
Caseinolytic	0.006 ± 0.003

[a]Subscript indicates the time during the incubation when OD405 was recorded for the standards and ECM.
[b]OD405was computed as a mean from OD405 recorded at 30, 60, 90 and 120 min of incubation for each standard and ECM.

Discussion

Hatching from the zona pellucida is a necessary process for an embryo to go through if it is going to survive and this is a good indicator of embryo viability. It would be beneficial in an embryo transfer procedure to accurately predict which embryos are going to hatch and generate viable pregnancies because embryos are transfered as blastocysts before hatching. One way to predict an embryo's likelihood of hatching would be to determine the amount of uPA present in conditioned medium recovered from a blastocyst (Kaaekuahiwi and Menino, 1990).

Urokinase-type plasminogen activator is a serine protease which cleaves the zymogen plasminogen into the active enzyme plasmin. The tripeptide EGR is a synthetic substrate for uPA and is commonly used in colorimetric assays. Dipsticks constructed with EGR enabled visual detection of enzymatic cleavage by 10 and 100 IU/mL UK but were not sensitive enough to detect uPA in ECM.The 96-well plate technique was successful in detecting uPA in ECM after 30 min of incubation but only photometrically.

These data demonstrate it is possible to rapidly quantify the amount of uPA being produced by an embryo using the 96-well plate assay. However, to use the plate-based assay would require the embryo transfer practitioner to have an ELISA plate reader on hand and the expense associated with such an instrument may limit its on farm application.

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CHAPTER 5

CONCLUSION

For Experiment 1, although cows superovulated with 400 mg FSH and 100 μg GnRH (the standard doses) produced more total ova, many were either unfertilized or, if fertilized, not transferable embryos. Embryos recovered from cows superovulated with 200 mg FSH developed to more advanced cell stages and produced more plasminogen activator than embryos recovered from cows treated with 400 mg FSH. A higher percentage of transferable embryos was collected from cows superovulated with 200 mg FSH than from cows superovulated with 400 mg FSH. Higher percentages of unfertilized ova and degenerate embryos were collected from cows superovulated with 400 mg FSH compared to cows superovulated with 200 mg FSH. Also, more unfertilized ova were collected from cows superovulated with 200 μg GnRH than from cows superovulated with 100 μg GnRH. Similarly, in Experiment 2, cows superovulated with 400 mg FSH also produced more total ova than cows superovulated with 200 mg FSH, but a higher number of these ova were unfertilized or degenerate. Additionally, when cows treated with 400 mg FSH were superovulated a second time, the percentage of degenerate embryos was almost twice as high as the first round.

These data suggest that the higher dosing is likely inducing ovulation of poorer quality ova which either fail to fertilize or, if fertilization occurs, fail to generate competent embryos. Producers using embryo transfer in their herds should consider using reduced dosages of FSH for superovulating donor cows because embryo viability after collection is compromised with the higher doses. One strategy may be to start donors on a reduced dose of FSH, then adjust to a higher dose as the superovulatory response is

observed to decrease. The reduced FSH dose not only contributes to a reduced cost but also extends the longevity of a cow to serve as a donor.

In Experiment 3, visible color was only discerned in wells containing EGR and 100 IU urokinase/mL after a minimum of 30 mins, although optical densities, as measured by an ELISA plate reader, were distinguishable between all concentrations of urokinase, and optical densities were shown to increase as the concentration of urokinase increased. Additionally, optical densities sometimes increased within a particular treatment as incubation time increased, and yellow color observed in wells was shown to grow darker as incubation time increased. Visible color was also present on dipsticks incubated with 10 or 100 IU urokinase/mL.

These data show that it is possible to detect the presence and quantify the amount of plasminogen activator (PA) being produced by an embryo in a qualitative and quantifiable manner. The dipstick assays are a quick, easy method to use on-farm, but they are not quite sensitive enough to detect the lower levels of PA being produced by a developing embryo (between 0-10 IU/mL). The optical densities obtained from the wells of 96-well plates make this assay sensitive enough to detect the lowest levels of urokinase standard used (1 IU/mL), but an ELISA plate reader is necessary to obtain the data. Therefore, practitioners would need to have access to an ELISA plate reader when they are on site and performing embryo transfers, which is not very practical.

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Appendix 1

Dipstick Assays

1. Squares (5cm x 5cm) of cellulose acetate (CA), chromatography paper (CP), glass fiber membrane (GFM) and nitrocellulose (NC) were blocked in TTBS containing 3% BSA on a rocker for 1 h. In some experiments, the squares were treated with poly-L-lysine and incubated in a humidity box for 1 h prior to being blocked.

2. Squares were allowed to dry at 37^{o}C.

3. In some experiments, squares were incubated in 5 mL 0.5% glutaraldehyde phosphatase for 1 h, rinsed in TTBS on a rocker for 1 h, and allowed to dry at 37^{o}C.

4. The squares were cut into several smaller squares (1mm x 1mm), and 1 or 2μL of a capture antibody (Ab-2) was pipetted onto the squares. In some experiments, the capture antibody was pipetted directly onto a strip.

6. 1 μL of alkaline-phosphatase linked antibody (Ab-1) was placed onto an untreated 1mm x 1mm square of substrate, and these were attached to nitrocellulose or plastic substrate using superglue or double sided tape. See Fig. 23 for construction of a strip made of nitrocellulose and glass fiber membrane.

7. Antibodies were dried onto the substrates at 37^{o}C.

8. Strips were incubated in 25 μL of media containing 0, 1, 10 or 100 IU urokinase/mL or cultured embryo media until all media had wicked up the strips.

9. Strips were rinsed in TTBS on a rocker for 15 mins, and TTBS was changed every 5 mins.

10. A kit (Bio-Rad, United States, cat #170-6432) was used to develop the strips so that Ab-1 would turn purple, allowing the results of the strips to be read.

11. Strips were allowed to dry at 37^{o}C before being preserved in a notebook.

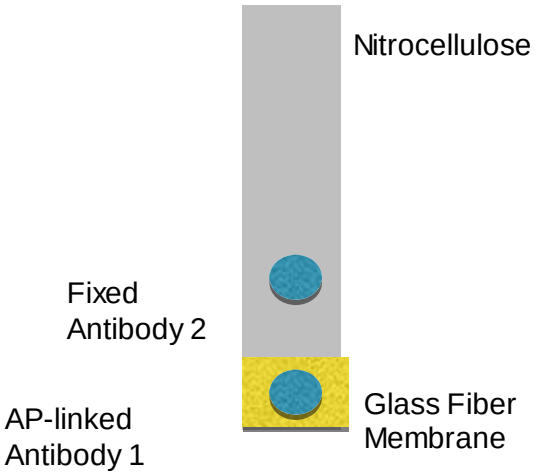

Figure 23. The construction of a double antibody dipstick assay.

The following trials were conducted and results were tabulated (Table 9).

Table 9. Visual detection of primary and secondary antibodies in plasminogen activator double antibody assays.

Main Substrate	Secondary Substrate	[UK]	Primary AB visible (Y= yes, N= no)	Secondary AB visible (Y=yes, N=no)
NC	NC	1	Y	N
NC	NC	1	Y	N
NC	NC	1	Y	N
NC	NC	1	Y	N
NC	NC	0	Y	N
NC	NC	0	Y	N
NC	NC	1	Y	N
NC	FP	1	Y	N
NC	NC	0	Y	N
NC	FP	0	Y	Y
NC	NC	1	Y	N
NC	FP	1	Y	N
NC	NC	0	Y	N
NC	FP	0	Y	N
NC	FP	0	Y	N
NC	FP	0	Y	N

Table 9. Visual detection of primary and secondary antibodies in plasminogen activator double antibody assays. (Continued)

NC	FP	10	Y	N
NC	FP	10	Y	N
NC	FP	100	Y	N
NC	FP	100	Y	N
NC	FP	1000	Y	N
NC	FP	1000	Y	N
NC	NC	0	N	N
NC	NC	0	N	Y
NC	NC	10	N	Y
NC	NC	10	N	Y
NC	NC	100	N	Y
NC	NC	100	N	Y
NC	NC	1000	N	N
NC	NC	1000	N	Y
NC	FP	0	N	N
NC	FP	0	N	N
NC	FP	1	N	N
NC	FP	1	N	N
NC	FP	10	N	N
NC	FP	10	N	N
NC	FP	100	N	N
NC	FP	100	N	N
NC	FP	0	Y	N
NC	FP	0	Y	Y
NC	FP	1	N	N
NC	FP	1	N	N
NC	FP	10	N	N
NC	FP	10	N	N
NC	FP	100	N	N

Table 9. Visual detection of primary and secondary antibodies in plasminogen activator double antibody assays. (Continued)

NC	FP	100	N	N
GFM	FP	0	Y	N
GFM	FP	0	Y	N
GFM	FP	1	Y	N
GFM	FP	1	Y	N
GFM	FP	10	Y	N
GFM	FP	10	Y	N
GFM	FP	100	Y	N
GFM	FP	100	Y	N
GFM	NC	0	Y	N
GFM	NC	1	Y	N
GFM	NC	10	Y	N
GFM	NC	100	Y	Y
GFM	NC	0	Y	N
GFM	NC	1	Y	Y
GFM	NC	10	Y	Y
GFM	NC	100	Y	N
GFM	CP	0	Y	N
GFM	CP	1	Y	N
GFM	CP	10	Y	N
GFM	CP	100	Y	N
GFM	CP	0	Y	FAINT
GFM	CP	1	Y	Y
GFM	CP	10	Y	Y
GFM	CP	100	Y	Y
GFM	CP	0	Y	Y
GFM	CP	1	Y	N
GFM	CP	10	Y	N
GFM	CP	100	Y	Y

Table 9. Visual detection of primary and secondary antibodies in plasminogen activator double antibody assays. (Continued)

GFM	CP	0	Y	N
GFM	CP	1	Y	N
GFM	CP	10	Y	N
GFM	CP	M	Y	N
GFM	CP	0	Y	Y
GFM	CP	1	Y	N
GFM	CP	10	Y	N
GFM	CP	100	Y	FAINT
GFM	NC	0	Y	FAINT
GFM	NC	1	Y	FAINT
GFM	NC	10	Y	Y
GFM	NC	100	Y	Y
GFM	NC	0	Y	FAINT
GFM	NC	0.1	Y	FAINT
GFM	NC	1	Y	FAINT
GFM	NC	10	Y	Y
GFM	NC	100	Y	Y
GFM	NC	0	Y	Y
GFM	NC	0.1	Y	Y
GFM	NC	1	Y	Y
GFM	NC	10	Y	Y
GFM	NC	100	Y	Y
GFM	NC	0	Y	FAINT
GFM	NC	0.1	Y	Y
GFM	NC	1	Y	FAINT
GFM	NC	10	Y	FAINT
GFM	NC	100	Y	FAINT
GFM	NC	0	Y	N
GFM	NC	1	Y	FAINT

Table 9. Visual detection of primary and secondary antibodies in plasminogen activator double antibody assays. (Continued)

GFM	NC	10	Y	N
GFM	NC	100	Y	Y
GFM	NC	0	Y	N
GFM	NC	1	Y	N
GFM	NC	10	Y	N
GFM	NC	100	Y	N
GFM	NC	0	Y	Y
GFM	NC	1	Y	N
GFM	NC	10	N	Y
GFM	NC	100	Y	FAINT
GFM	NC	0	N	N
GFM	NC	1	Y	FAINT
GFM	NC	10	Y	Y
GFM	NC	100	Y	Y
GFM	NC	0	Y	Y
GFM	NC	1	Y	Y
GFM	NC	10	Y	FAINT
GFM	NC	100	Y	N
GFM	NC	0	Y	Y
GFM	NC	1	Y	Y
GFM	NC	10	Y	Y
GFM	NC	100	Y	Y
GFM	NC	0	Y	FAINT
GFM	NC	10	Y	N
GFM	NC	M	Y	FAINT
GFM	NC	0	Y	N
GFM	NC	1	Y	Y
GFM	NC	10	Y	N
GFM	NC	100	Y	N

Table 9. Visual detection of primary and secondary antibodies in plasminogen activator double antibody assays. (Continued)

NC	NC	0	Y	FAINT
NC	NC	10	Y	Y
NC	NC	10 + 1000 µL TTBS	Y	Y
GFM	GFM	0	N	N
GFM	GFM	10	N	N
GFM	GFM	10 + 1000 µL TTBS	FAINT	FAINT
CP	CP	0	Y	N
CP	CP	10	Y	N
CP	CP	10 + 1000 µL TTBS	Y	N
CA	CA	0	Y	N
CA	CA	10	Y	N
CA	CA	10 + 1000 µL TTBS	Y	N
GFM	CP	0	N	N
GFM	CP	1	Y	FAINT
GFM	CP	10	N	N
GFM	NC	100	Y	Y
GFM	NC	0	Y	N
GFM	NC	1	Y	FAINT
GFM	NC	10	Y	N
GFM	NC	100	N	Y
GFM	NC	0	Y	Y
GFM	NC	1	Y	Y
GFM	NC	10	Y	Y
GFM	NC	100	Y	Y
GFM	NC	0	Y	Y
GFM	NC	1	Y	Y
GFM	NC	10	Y	Y
GFM	NC	100	Y	Y
GFM	NC	0	Y	Y

Table 9. Visual detection of primary and secondary antibodies in plasminogen activator double antibody assays. (Continued)

GFM	NC	1	Y	N
GFM	NC	10	Y	N
GFM	NC	100	Y	N
GFM	NC	0	N	N
GFM	NC	1	Y	N
GFM	NC	10	Y	FAINT
GFM	NC	100	Y	FAINT
GFM	NC	0	Y	FAINT
GFM	NC	1	Y	FAINT
GFM	NC	10	Y	N
GFM	NC	100	Y	Y
GFM	NC	0	Y	N
GFM	NC	1	Y	N
GFM	NC	10	Y	Y
GFM	NC	100	Y	N
GFM	NC	0	Y	FAINT
GFM	NC	1	Y	FAINT
GFM	NC	10	Y	Y
GFM	NC	M	Y	FAINT
GFM	NC	0	Y	FAINT
GFM	NC	1	Y	Y
GFM	NC	10	Y	FAINT
GFM	NC	M	Y	FAINT
GFM	GFM	0	Y	N
GFM	GFM	1	Y	FAINT
GFM	GFM	10	Y	N
GFM	GFM	M	Y	N
GFM	GFM	0	Y	N
GFM	GFM	1	Y	N

Table 9. Visual detection of primary and secondary antibodies in plasminogen activator double antibody assays. (Continued)

GFM	GFM	10	Y	FAINT
GFM	GFM	M	Y	FAINT
GFM	GFM	0	Y	N
GFM	GFM	1	Y	N
GFM	GFM	10	Y	N
GFM	GFM	M	Y	N
GFM	GFM	0	Y	N
GFM	GFM	1	Y	N
GFM	GFM	10	Y	N
GFM	GFM	M	Y	N
GFM	GFM	0	Y	N
GFM	GFM	1	Y	N
GFM	GFM	10	Y	N
GFM	GFM	M	Y	N
GFM	GFM	0	Y	N
GFM	GFM	1	Y	N
GFM	GFM	10	Y	N
GFM	GFM	M	Y	N