LHRH FUSION PROTEIN VACCINES IN BEEF HEIFERS AND BOVINE ECTOPIC OVARIAN XENOGRAFTING

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the dissertation of JEFFREY DAVID STEVENS find it satisfactory and recommend that it be accepted.

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Abstract

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The objectives of the studies herein are two-fold. First is to evaluate immune and biological responses from alternating two fusion proteins in an immunization schedule. A carrier-mediated immune suppression has been reported in the literature when immunizing with the same carrier repeatedly. We want to bypass this carrier-mediated immune suppression by using two different carriers in an immunization schedule. One hundred and ten heifers were divided equally into 11 treatment groups. Control groups were either spayed or intact non-treated animals. Heifers in the other nine groups were immunized on wk 0, 4 and 9. Treatments were immunizations of the same protein throughout or alternating the proteins in different booster sequences. Blood was collected for measurement of reproductive hormones and antibody production. Reproductive tracts were collected at time of slaughter. Anti-LHRH antibodies were detected in all immunized groups by the sixth wk of the study while the two control groups of heifers did not have detectable anti-LHRH antibodies at any time during the study. The number of heifers cycling in the immunized groups was decreased compared to the intact control group by wk 6 and these treatment groups continued to show suppression of estrous cycle activity through the conclusion of the study. No difference

in number of heifers cycling between immunized groups and the spayed heifers was found during wk 9 to 22. The second objective was to examine the possibility of a xenograft system for bovine ovarian cortical tissue that would allow for normal follicular development to occur in the host animal. Thirty-two nude mice were used as recipient animals for bovine ovarian cortical tissue from either adult animals or d 160 fetal calves. The number of follicles in fetal ovarian tissue was much greater than in adults prior to grafting. Ovarian tissue did not develop beyond the early secondary stage in any of the mouse grafts and much of the ovarian tissue developed fibrosis. We were unable to localize follicle stimulating hormone receptor to any of the grafted tissue with the exception of d 160 fetal ovaries that had been grafted for 2 wk.

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REVIEW OF LITERATURE

INTRODUCTION

Over the past 60 years understanding of reproduction has enhanced the ability to control the reproductive cycle in cattle. Attention has focused primarily on suppressing the estrous cycle, or stimulating it and much of the current knowledge was attained from studies involving alterations of hormones in cattle. Most notable has been the ability to suppress estrus in heifers in feedlots, equally important is the ability to synchronize estrous cycles using exogenous hormones.

More recently there has been increasing interest in suppressing estrus in cattle prior to their entering the feedlot. Under range conditions, this has been a major problem for the industry because feeding a supplement such as MGA is not possible. This is a big concern because approximately 12 million heifers are fed in the feedlot each year; of these approximately 7% are pregnant at the time they enter the feedlot (USDA, 2000). Since these pregnant animals are putting energy from feed into pregnancy and growth of the fetus, they are not gaining weight at an optimum level and are therefore costing the industry money. It is estimated that the cost of these animals entering the feedlot is $200 million per year. For this reason, as well as the decreased feed conversion (Kreikemeier and Unruh, 1993) and decreased final weight, all female cattle entering the feedlot are purchased at a reduced cost.

If the feedlot industry could be guaranteed that females coming into their facility were not pregnant, feeders would potentially pay more for them. One method to inhibit pregnancy and estrous cycles would be to immunize these cattle against luteinizing

hormone- releasing hormone (LHRH). This immunization would block LHRH from acting on the gonadotroph cells in the adenohypophysis, thus blocking release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) (Adams and Adams, 1986), effectively stopping the estrous cycle and inhibiting pregnancy. Although numerous groups demonstrated that active immunization against LHRH suppresses ovarian function in beef heifers, different success rates have been obtained thus far (Adams and Adams, 1990; Wettemann and Castree, 1994; and Prendiville et al., 1995).

Because the structure of LHRH is very similar between animal species, effective immunization requires LHRH to be coupled with a carrier to make it antigenic. One problem associated with coupling LHRH to a carrier is that it needs to be done the same way both within a batch and between batches of vaccine made. Without this consistency, this vaccine would not be approved for use by the FDA. Recently, two carrier/LHRH fusion proteins were developed. The first is comprised of seven LHRH molecules attached to ovalbumin and the second is seven LHRH molecules attached to *E. coli* thioredoxin. Both of these fusion proteins are being produced in a bacterial expression system to ensure consistent composition of this vaccine preparation.

Sterilization vaccines

Sterilization vaccines have been a topic of interest in recent years. With the increased cost associated with feeding pregnant animals in the feedlot, and the reduced gain from these animals, this becomes a major concern. Producers have several choices regarding these animals. The first option is to spay any females entering the feedlot. Unfortunately, this is a surgical procedure and is more expensive than the resulting

improvements in gains received. There is also a reduction in feed efficiency due to a lack of ovarian steroids. Second, producers could vaccinate their cattle against an endogenous hormone, disrupting the estrous cycle and therefore stopping pregnancy. In recent years, many different vaccines have been produced and tested in beef cattle.

Prostaglandin F2α

Sterilization vaccines have been developed that immunize an animal against Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). The theory being that the corpus luteum (CL) will be maintained (Scaramuzzi and Baird, 1976) and subsequent ovulation (Bettencourt et al., 1993), and therefore estrous behavior, will be blocked. Chang et al. (1987) found immunization with a $PGF_{2\alpha}$-ovalbumin conjugate to be effective in interrupting the normal sequence of events in the estrous cycle by neutralizing endogenous $PGF_{2\alpha}$. This was the first report that active immunization against $PGF_{2\alpha}$ results in sterilization through prolongation of the estrous cycle in beef cattle. That research determined that CL function was maintained for a minimum of 75 d, and ovulation during this time was blocked. It had previously been demonstrated that passive immunization with $PGF_{2\alpha}$ (Fairclough et al., 1981) prolongs the estrous cycle in cows and ewes.

Bettencourt et al. (1993), while trying to improve fertility in postpartum ewes utilizing a vaccine against $PGF_{2\alpha}$, found that ovulation was blocked in these ewes. Previously it was shown that ovulation occured in the absence of a preovulatory rise in ovarian $PGF_{2\alpha}$ (Murdoch, 1988). It was also suggested that products of the lipoxygenase pathway may be more important for ovulation than prostanoids (Tanaka et al., 1991). However, in the ewe, this could not be demonstrated since an inhibition of leukotriene

synthesis was unable to block ovulation (Tanaka et al., 1991). The fact that other researchers were unable to block ovulation in $PGF_2\alpha$ immunized ewes (Scaramuzzi and Baird, 1976) is potentially related to antibody titers. It is possible that titers were not high enough to block ovulation but were high enough to inhibit luteolysis.

Luteinizing Hormone

Vaccines against luteinizing hormone (LH) were first utilized in the 1960's when active immunization against LH was shown to cause a decrease in serum T in rabbits and rats (Wakabayashi and Tamoaki, 1966) and goats (Hayashida and Farook, 1969). Active immunization against LH was also shown to cause atrophy of the gonads and accessory sex organs of both male (Pineda et al., 1967) and female (Pineda et al., 1968) rabbits. Then, in 1985, Fitzgerald et al. treated adult Suffolk ewes with an anti-LH antiserum to determine its effect on reproduction. Interestingly, these authors found an increase in the ovulation rate (2.7 ± .2 vs 2.1 ± .1) over that of control ewes. It was shown that circulating concentrations of follicle stimulating hormone (FSH) were increased in treated ewes. There was also an increase in estrous cycle length and an overall increase in ovulation rate. A change in the LH to FSH ratio was implicated in increasing the ovulation rate (Fitzgerald et al., 1985). Interestingly, these researchers never explained how an animal that is immunized against the hormone responsible for ovulation in the ewe was able to ovulate. The only explanations I can ascertain is that the antibody to LH is not very specific, allowing for quite a bit of LH to still reach the ovary or the antibody was cleared from the body, allowing for ovulation to occur.

In 1985, Schanbacher reported a reduced gonadal weight and function in male cattle and sheep when using active immunization against LH. In contrast to the results of Fitzgerald et al. (1985), the antibody production to LH was most likely more specific, thus being more effective in blocking LH from having its action on the testis. In support of Schanbacher's results, several other authors (Falvo et al., 1986; Johnson et al., 1988; Grieger and Reeves, 1988; Roberts et al., 1990; Grieger and Reeves, 1990; Grieger et al. 1990) have found that active immunization against LH is effective in inhibiting reproductive performance.

Porcine Zona Pellucida

A vaccine against the zona pellucida protein has been developed for several years and is currently being used in many zoos to inhibit reproduction of large felines and other animals. The vaccine is prepared from pig ovaries, thus giving the name porcine zona pellucida (pZP) (Liu et al., 1989). Immunization with pZP results in zona antibody titers and fertility inhibition by blocking the sites for sperm recognition.

Other research being performed with the pZP vaccine involves vaccinating animals in the wild that are considered a nuisance. This would include studies on controlling wild deer, burro and horse populations (Kirkpatrick et al., 1997; Turner et al. 1997; Bastian et al., 1999).

A large glycoprotein, referred to as ZP3, has been identified to be the specific zona receptor for sperm recognition and attachment (Sacco et al., 1984). The pZP vaccine works through having antibodies bind the ZP3 protein on the oocyte, therefore tying this protein up and not allowing the sperm to bind or penetrate the oocyte, thus

blocking fertilization. According to Willis et al. (1994), in vitro experiments the pZP vaccine was found to be effective in blocking 99% of spermatozoa binding. Some reports (Skinner et al., 1984; Mahi-Brown et al., 1985; Dunbar et al., 1989) have found a possible altered ovarian function with repeated immunizations of pZP. According to Kirkpatrick et al. (1997), treatment with the pZP vaccine can be utilized for up to three consecutive years and still allow for a return to fertility. Willis et al. (1994) utilized a "biobullet" to deliver the vaccine to 4 mares for each of 2 years. It was determined that the biobullet was effective in blocking fertilization in all mares treated. Ovulation rates for mares treated from 1 to 7 years differ significantly however with those animals treated for 1 year having a 73% ovulation rate and those treated 7 years only having a 10% ovulation rate Kirkpatrick et al. (1997).

More recently Miller et al. (2000) has developed a recombinant rabbit ZP immunocontraception vaccine with the belief that gathering porcine ovaries from the slaughterhouse is not cost effective. While somewhat effective in decreasing the number of offspring from treated deer, these recombinant vaccines were not as effective as native pZP immunizations. It was believed that high antibody titers were developed to the carrier protein while there were low antibody titers to the recombinant protein. Similar problems have been assumed when using a vaccine against LHRH.

Luteinizing Hormone-Releasing Hormone

Recently, most researchers have stopped making vaccines to immunize against reproductive hormones with one large exception, luteinizing hormone-releasing hormone (LHRH). Over the past 24 years, a major interest in blocking cyclicity in female cattle

has been looked at primarily through the use of vaccines against the hormone that is thought of as the master controller of reproduction. Luteinizing hormone-releasing hormone is released from the hypothalamus in a tonic manner. However, under the influence of estrogen, pulses of LHRH are released from the surge center of the hypothalamus, these pulses cause the release of LH and FSH. Follicle stimulating hormone causes follicular growth while LH causes ovulation of dominant follicles.

If the pulsatile release of LHRH or the action of the pulses on the anterior pituitary could be blocked, there will be no follicular growth and subsequently, no release of oocytes from the follicles. Fraser and McNeilly (1983) found that injecting ovine antiserum to LHRH in ewes during the late follicular phase of the estrous cycle resulted in an immediate blockage of the pulsatile secretion of LH while the secretion of FSH gradually increased for the first 36-h of the study period. These results indicate that pulsatile LH secretion is dependent upon LHRH release from the hypothalamus while there is either another FSH-releasing hormone or the release of FSH is not only dependent upon LHRH. Similar results were found by other authors (Culler and Negro-Vilar, 1987), suggesting FSH release may be under the control of not just LHRH, but some other factors, while pulsatile LH release can be effectively blocked by blocking the action of LHRH on the gonadotroph cells in the pituitary.

Culler and Negro-Vilar (1987) suggest that pulsatile FSH secretion from the pituitary is spontaneous or, more likely, that there is an endogenous FSH-releasing factor that is distinct from LHRH, which stimulates the pituitary to release FSH in a pulsatile manner. It is suggested that LHRH may act permissively or synergistically with an unknown FSH-RH to elevate mean plasma FSH (Culler and Negro-Vilar, 1987). It was

concluded (Culler and Negro-Vilar, 1987) that LHRH affects both LH and FSH secretion, however LHRH cannot be considered the sole physiological regulator of FSH secretion. It is important to note that injection of LHRH antibodies prior to the preovulatory gonadotrophin surges in the rat and ewe will prevent the surge of FSH as well as LH (Arimura et al., 1974; Fraser and McNeilly, 1982), indicating that the surge of FSH relies on the release of LHRH. It was hypothesized (Fraser and McNeilly, 1983) the maintenance of plasma FSH levels during immunization with antibodies to LHRH may be due to several factors including, 1) autonomous release of FSH, 2) incomplete nutralization of LHRH, 3) an FSH-RH, or 4) differential secretion of ovarian factors that act directly on the pituitary gonadotrophs during immunization. The ability of an LHRH sterilization vaccine to block both LH and FSH, or just LH does not appear to matter. In either case, blocking the LH surge will be enough to block ovulation.

After demonstrations of short-term immunonutralization on the effects of LHRH (Fraser and McNeilly, 1983), the effects of long-term immunocastration were evaluated in the ewe (McNeilly et al., 1986). Immunizations were performed by conjugating LHRH to human serum albumin and were given to normally cycling ewes in week 1, followed by boosters at weeks 21, 39, 56 and 79, the study was concluded after 22 months. The authors found plasma FSH levels decreased within 3 weeks of the primary immunization and to remain suppressed for the remainder of the study. This finding is in contrast to those of Clarke et al. (1978) and Fraser and McNeilly (1983). There was a low level of FSH detected in the plasma (McNeilly et al., 1986) that may have resulted in either the intrinsic ability of the sheep pituitary to produce FSH or from incomplete abolishment of LHRH release from reaching the pituitary.

Shally (1971) determined that the synthetic decapeptide (LHRH) could stimulate the release of not only LH, but also FSH both *in vitro* and *in vivo*. This would suggest there was incomplete abolishment of LHRH release in the study by McNeilly et al. (1986). In contrast to the findings of Shally (1971), it was demonstrated (Culler and Negro-Vilar, 1986) that pulsatile FSH secretion is independent of endogenous LHRH but that LHRH is required to maintain elevated plasma FSH, again suggesting there is some other mechanism responsible for the release of FSH. McNeilly et al. (1986) noted that LH secretion did not change during the study period, indicating an absence of pulsatile secretion.

Culler and Negro-Vilar (1987) found complete blockage of pulsatile LH secretion and a mean plasma LH reduction by 86.4% when compared to control rats treated with non-immune serum. However, pulsatile FSH secretion was not affected in this study even though during the fourth hour of collection, mean basal levels were lowered by an average of 58% compared to non-immune serum treated rats.

Follicular Development as a result of immunization against LHRH

Immunization against LHRH was shown to prevent normal follicular development with a complete lack of histologically healthy follicles greater than 2 mm in diameter in ewes (McNeilly et al., 1986). Johnson et al. (1988) noticed a similar trend in cattle. Follicular development beyond 5 mm did not occur in heifers that responded to the LHRH vaccine in their study. McNeilly et al. (1986) noted an increase in atretic follicles between 1 and 2 mm in diameter in immunized sheep when compared to control

sheep even though there were more follicles in the 1 to 2 mm size group in immunized ewes. Similar results have been found in cattle (Prendiville et al., 1995).

In ewes, LH is required in a pulsatile manner for follicles to grow beyond 3 mm, this is presumably to generate production of androgens from the theca for subsequent conversion of estradiol by the granulosa cells (Baird et al., 1981; McNeilly et al., 1984). This is similar to the results found after hypophysectomy (Dufour et al., 1979). These results may be due to the low release of FSH in their experiment. A low release of FSH will sustain follicular growth to a point but will not be enough to ensure continued growth of the follicle. However, this does not explain the similarities of follicular growth after hypophysectomy, it may be that the initial growth of follicles does not require any FSH release from the pituitary, but once follicles reach a certain size, their continued growth and survival becomes dependent upon FSH and LH. Another possibility for the increase in atretic follicles when ewes were vaccinated is that each follicle is exposed to less androgen and therefore the final stages of atresia, assuming this is an androgen dependent process, may proceed at a reduced rate, thus resulting in an accumulation of atretic follicles in the ovary (Baird et al., 1981).

Bishop et al. (1996) studied the effects of follicular development after immunization against LHRH, however these authors wanted to determine if they could induce cycling with an exogenous treatment of gonadotropins. These authors found similar results as others (McNeilly, 1986; Prendiville et al., 1995); immunization against LHRH blocked the LH surge and therefore blocked ovulation, unless the animal was given an exogenous source of LH and FSH. While this demonstrates that ovarian control can be altered under this regimen, one thing this does not address is the fact that

gonadotropin support would need to be given continuously for this animal to maintain pregnancy. Johnson et al. (1988) found fewer follicles and CL in heifers immunized against LHRH, similar to Bishop et al. (1996).

Reproductive tract effects of immunization

Immunization against LHRH will decrease the amount of gonadotropins in circulation, thus decreasing estrogen. Since reproductive tract development weight and development is in part dependent upon estrogen it would make sense that animals immunized against LHRH would have lighter reproductive tract weights. In 1990, Adams and Adams reported a reduced ovarian and uterine weight in immunized heifers when compared to non-immunized heifers. Similarily, Johnson et al. (1988) noted that the ovarian weight was lower in LHRH immunized heifers when compared to their non-immunized counterparts. Not only did Adams and Adams (1990) find the decreased reproductive tract weights, they also found a decrease in the LH concentration and the LHRH receptor number in the anterior pituitary.

Anti-LHRH antibodies

For adequate suppression of estrus, anti-LHRH antibodies must be developed against LHRH. Prendiville et al. (1995) found a positive correlation between anti-LHRH antibodies and duration of anestrus. The higher the antibody production, the longer the anestrous interval in their study. These results support those of Wettemann and Castree (1994), who found the onset of puberty to be closely related to the magnitude of anti-LHRH antibodies present following immunization against LHRH in prepubertal heifers.

Reports by Bell et al., (1997) indicate that while high levels of anti-LHRH antibodies may be evident in most immunized heifers at the beginning of a breeding period, it appears that some heifers are insensitive to the LHRH vaccine, thus allowing these heifers to get pregnant prior to their entering the feedlot. Similarily, Adams and Adams (1992) reported a lack of correlation between anti-LHRH antibody level and biological immunity. Finnerty et al. (1994) claimed some evidence suggesting anti-LHRH antibodies are not biologically active after they reach a plateau, however, no other data is reported to substantiate this. Gual et al. (1997), when immunizing post-menopausal women with a LHRH vaccine, found that anti-LHRH antibodies remained high enough for approximately 180 days to reduce serum LH and FSH. However, without further booster immunizations, the effects of the vaccine were blocked. They claim that the level of antibodies decreased to a point below the bioeffective level, therefore allowing for an increase in LH and FSH. While no statistics accompany this claim and only visual appraisal of graphs is apparent, it appears that this claim may not be true. In two of the three subjects, the anti-LHRH antibodies were at least level if not still increasing in this study.

In most cases, researchers give a primary immunization followed by one or more booster immunizations at set time intervals. It is believed that at least one booster is required to develop antibody titers high enough to affect reproductive performance. However, in 1992, Adams and Adams found that the pattern of antibody development and the maximal anti-LHRH antibody production were not different between steers receiving either a single immunization alone or a primary and secondary immunization.

Feedlot performance of immunized heifers

During confinement, suppression of ovarian activity may be used as a management tool to reduce the effects of recurrent estrus such as reduced intake and a decrease in mounting activity (O'Brien et al., 1968). Suppression of ovarian activity prior to entering the feedlot can be attained using either surgical or immunological methods, which would reduce the incidence of pregnancy among these heifers. Ovariectomy and hysterectomy are impractical since they can cause morbidity and mortality and decrease animal performance (Zinn et al., 1989).

The feedlot performance of ovariectomized heifers and heifers actively immunized against LHRH have been found to be lowered when compared to intact heifers (Adams and Adams, 1990). A subsequent study by Adams et al. (1990) was performed to determine if treatment with an anabolic steroid (Synovex-H) would allow for increased growth rates of immunized heifers. Immunized, non-implanted heifers had a significant decrease in weight gains when compared to control heifers. However, the administration of Synovex-H combined with immunization resulted in weight gains that were comparable to the total gain in control heifers with Synovex-H implants, indicating that the decrease in total steroids due to immunization can be overcome with an implant when heifers enter the feedlot. Prendiville et al. (1995) found in contrast to results of Adams and Adams (1990), that final live weight and average daily gain (ADG) were not different in control versus vaccinated animals over the entire study. However, it was noted that immunized heifers had a reduced ADG between days 56 and 84 versus control animals. Thus, immunized heifers in this study did not require a steroid implant to be as efficient as control animals.

When comparing the carcass characteristics of LHRH immunized heifers to heifers that were non-immunized and bull exposed and heifers that were non-immunized it was determined that the immunized animals had an intermediate dressing percentage between the other two groups with the non-immunized and bull exposed group being the lowest (Bell et al., 1997). All heifers in this study received a growth implant at entry into the feedlot. It was noted that additional trimming was required at the site of immunization thus leading these researchers to believe that the dressing percentage should have been slightly higher for this group of animals.

Testicular development

Not only does immunization of heifers offer a promising method of castration versus surgical methods, but immunization of bulls may become the method of choice for castration in the future. In countries that have large problems with screw worms or other flies, surgical castration of cattle is rarely used due to the levels of mortality following such a procedure. Reports indicate (Adams et al., 1996) that LHRH immunized bulls have a decrease in the masculinity of the carcass versus their unimmunized contemporaries. This leads to a carcass that is more acceptable to the consumer. It was also found (Adams et al., 1996) that feedlot gain was intermediate to unimmunized bulls and unimplanted steers. However, in 1992 Adams and Adams indicated no difference in either final live weight or ADG between unimmunized bulls and those bulls immunized against LHRH. Thus, feedlot performance appears not to be affected by immunizing bulls against LHRH and appears to be a non-invasive alternative to physical castration in the management of livestock. It is important to note that while feedlot gain may not

differ, yield grade has been shown to be better in steers than in bulls either immunized or unimmunized against LHRH. However, in some countries, feeder bulls are not placed in the feedlot, rather they are finished on grass. Since testosterone and estrogen are steroidogenic in cattle, it is in the producers interest to allow these grass finished animals to keep their testicles until shortly before slaughter. Prior to slaughter, immunization with LHRH or castration will allow the carcass to have a decreased masculinity, therefore becoming a better eating experience. According to Cook et al. (2000) responses to immunization appear to be optimal when the immunization occurred prior to puberty. Therefore, these grass finishers will have to either take the decrease in carcass value for some bulls due to the vaccination not working, or vaccinate earlier and take a decrease in growth rate.

Cook et al. (2000) found total daily sperm production to be severely reduced in immunized bulls, but the reduction in daily sperm production per gram of testicular parenchyma did not differ between LHRH immunized and control bulls. In part this lack of difference was probably due to the dramatic variation in the immunized bulls. On average, daily sperm production was about ½ that of control bulls when bulls were immunized against LHRH. Unfortunately, these bulls were not fertility tested, nor were they placed with fertile cows to determine whether they were able to impregnate these females. It is important to note that over the last 10 to 15 years, there appears to be a 25% increase in the ability of LHRH vaccinations to suppress serum testosterone concentrations in LHRH immunized bulls.

Reversibility of Immunization

Very little research has been performed aiming at the reversibility of an LHRH sterilization vaccine. It is known that exogenous gonadotropin stimulation will allow for follicular development and ovulation, however, can heifers resume normal estrous cycles if not given booster immunizations? According to D'Occhio et al. (1992) and Prendivelle et al. (1995), immunization against LHRH is reversible. Adams et al. (1996) indicated that bulls receiving a single immunization against LHRH were unable to maintain high antibody titers for 10 to 12 months. While these researchers did not test these bull calves for their ability to produce normal sperm, it may be assumed that a decrease in antibody titers will allow for LHRH to reach the anterior pituitary and thus cause the release of LH and FSH, allowing for normal spermatogenesis. For producers planning to send heifers to the feedlot, immunization prior to puberty may, in the future, ensure a premium since these heifers will be guaranteed to be not pregnant. However, in the case of some sort of natural disaster, some of these vaccinated heifers may be retained as replacement heifers without any damage to their reproductive performance (Prendivelle et al., 1995).

In 2001, D'Occhio et al. indicated that a transient active immunization against LHRH can potentially induce permanent suppression of reproductive function in bulls. Similar results have been seen in sheep (Brown et al., 1994 and Clarke et al., 1998) and swine (Molenaar et al., 1993). It appears that pathological changes in the median eminence including edemas, hypertrophy of magnocellular neurons and infiltration of fibroblasts occurs following long term antibody titers against LHRH that are high. The LHRH secreting neurons terminate outside the blood-brain barrier, thus it would make sense that antibodies would eventually attack this area. It was noted that the mean

gonadotropin concentrations in rams that had been immunized were lower than control animals, even after the anti-LHRH antibodies were undetectable (Brown et al., 1994) similar results were found in the ewe (Brown et al., 1995). It was hypothesized that LHRH immunization resulted in either reduced sensitivity of the pituitary to LHRH stimulation, or a diminished capacity of the pituitary to produce and secrete the gonadotrophs (Brown et al., 1995). A third alternative that was not mentioned in this paper is a reduced ability of the hypothalamus to either produce or secrete LHRH. It is possible that antibodies against LHRH attacked the area where LHRH is secreted, causing damage to the tissue and reducing the ability of that tissue to secrete hormone.

While immunizing humans and animals, there are reports of a carrier mediated immune suppression. More importantly, when using a LHRH sterilization vaccine in mice, Sad et al. (1991) reported a carrier mediated immune suppression to the LHRH vaccine.

Immunocontraceptives

How do immunocontraceptives work? To fully understand this question, one first needs to have a general understanding of the immune system. There are two types of immunity: innate immunity and acquired immunity. Innate immunity is something that is always there, animals are born with this type of immunity and don't have to be exposed to an antigen for this type of immunity to be acquired. Innate immunity is comprised of natural barriers, these would include the skin, low pH on the skin, and enzymes secreted in the saliva, mucus, and tears; phagocytes (cells that engulf antigens and break them into small pieces); and complement (proteins that have the ability to lyse a cell).

Acquired immunity is the type of immunity that is important for the immunocontraceptive to work. Acquired immunity is much more complex than innate immunity but it is also much more specific. It involves many different components of the immune system. The body develops a defense to a specific antigen and will remember exactly what that antigen looked like so that if encountered again a response can be immediate. While there is a certain amount of memory in acquired immunity, it is not permanent and will decrease over time. More detail about the immune system will help foster an understanding of how acquired immunity works.

The immune system is comprised of many components. Most immune cells are produced in the hematopoietic tissue of the long bones in the body. Contained in the bone marrow are a marked number of hematopoietic stem cells that are pleuripotent. These stem cells differentiate into several different types of cells: myeloid progenitor cells, which give rise to neurtophils, monocytes, Langerhan's/Kupffer cells, natural killer cells, eosinophils, mast cells, lymphoid progenitor cells, which give rise to T and B lymphocytes, megakaryocytes, and erythroblasts. For the most part, these cells remain undifferentiated until they are needed.

Of all these different cell types, two of them are extremely important for the success of immunocontracepives. These are the lymphocytes (B and T cells) and antigen presenting cells (APC's). Antigen presenting cells roam the body searching for any item seen as non-self, this would be an antigen. When APC's encounter antigens, they phagocytose them, then present fragments of the antigen on their surface. The APC's will eventually travel to a lymph node where they will be exposed to naïve B cells. The B cells will then begin to produce proteins known as antibodies that are specific to the

portion of antigen presented from the APC. The first antibodies produced are of the IgM classification of antibody.

B lymphocytes mature within the bone marrow, where they develop the ability to recognize a specific antigen. The B-cell receptor is a membrane-bound antibody molecule that is considered naïve until it encounters an antigen. When an antigen is encountered that matches its membrane bound antibody it causes the cell to rapidly divide giving off progeny that differentiate into memory B-cells and effector B-cells called plasma cells. Memory B-cells continue to express the same membrane-bound antibody as their parent naïve B-cell and they live longer than the naïve B-cell. On the other hand, plasma cells do not express a membrane bound antibody, rather they produce the antibody in a form that can be secreted. While plasma cells do not live very long, they secrete an enormous amount of antibody during their short life. These secreted antibodies are the largest effector molecules of humoral immunity.

T lymphocytes arise in the bone marrow similarly to B-cells. However, unlike B-cells which mature in the bone marrow, T-cells migrate to the thymus gland to mature. The T-cell comes to express a unique antigen-binding molecule called the T-cell receptor during its maturation within the thymus. T-cell receptors can only recognize antigen that is bound to cell-membrane proteins called major histocompatibility complex (MHC) molecules. When a naïve T-cell encounters antigen that is attached to a MHC molecule on a cell, the T-cell proliferates and differentiates into a memory T-cell and various effector T-cells.

There are 2 well defined subpopulations of T-cells: T helper (T_H) and T cytotoxic (T_C) cells. A third subpopulation, the T suppressor (T_S) cell, bears mentioning as some

researchers believe it exists as a separate population of cells while other researchers believe it may not be distinct from the T_H and T_C subpopulations. T cytotoxic and T_H cells can be distinguished from one another by the presence of a membrane bound glycoprotein on their surface. T-cells displaying the CD4 glycoprotein generally function as T_H cells, whereas those displaying the CD8 glycoprotein generally function as T_C cells.

After a T_H cell recognizes an antigen associated with a MHC class II molecule, the cell is activated and becomes an effector cell that secretes various cytokines. These cytokines play an important role in interacting with and activating B and T_C cells along with a host of other immune cells. The T_C cells that recognize an antigen, MHC class I molecule complex proliferate and differentiate into effector cells called cytotoxic T lymphocytes (CTL's). The CTL does not secrete many cytokines, rather they exhibit cytotoxic activity.

Activation of the humoral (termed because immunity is mediated by antibodies contained in body fluids) and cell-mediated immunity (termed because it appeared that cells, rather than serum components, were the major effectors in the immune response) system requires cytokines produced by T_H cells. These T_H cells can only recognize antigen that is displayed together with class MHC II molecules on the surface of APC's, thus their regulation is very strict. Antigen presenting cells must internalize antigen by either endocytosis or phagocytosis, break up the antigen and then display a portion of that antigen bound to a class II MHC molecule. The T_H cell can then recognize and interact with the antigen-class II MHC molecule complex allowing for activation of the T_H cell.

Carrier-Mediated Immune Suppression

Carrier-mediated immune suppression occurs when a host is repeatedly immunized with a hapten linked to an immunogenic carrier (Herzenberg and Tokuhisa, 1982). Typically, a strong secondary antibody response to the carrier is induced, but the animal does not produce an IgG response to the linked hapten epitope (Herzenberg et al., 1980; Herzenberg and Tokuhisa, 1982; Schutze et al., 1985). It was demonstrated (Sun and Wastenbaugh, 1986) that this regulatory system not only suppresses an antibody response against low molecular weight molecules, but also suppresses responses against immunogens of macromolecular weight. Moreover, it was demonstrated that such a regulatory system not only affects the antibody response to foreign antigens, but also influences the antibody response to a self-antigen (Rauch et al., 1981). Interestingly, while some studies have confirmed that preimmunization with a carrier can often result in inhibition of the production of antibodies to new epitopes liked to the same carrier (Herzenberg and Tokuhisa, 1982; Herzenberg et al., 1983; Schutze et al., 1985; John et al., 1989), other research shows that presensitization with a given carrier does not result in the suppression of responses, rather an enhancement (Lise et al., 1987). It has also been determined that in mice, not all strains are susceptible to this hapten-specific suppression. Therefore, it is believed that when immunizing a new species with a hapten-carrier conjugate, carrier-mediated immune suppression is given much thought and is tested accordingly.

Heal et al. (2001) discussed the importance of the cellular immune responses generated by T cells of both CD4+ and CD8+ subsets. These subsets can typically be classified as either type 1 or 2 as determined by the distince cytokine profile of the

antigen-specific T cells which produce them. For instance, type I responses are characterized by IL-2 and IFN-γ and promote the production of IgG2a opsonising and complement-fixing antibodies. In contrast, type II responses feature IL-4, IL-5, IL-10 and IL-12, and provide help for B cell maturation and the production of IgG1 and IgE. Thus, T-cells secreting cytokines broadly effect the cell-mediated and humoral immunity.

It was previously demonstrated by *in vivo* transfer that carrier-specific T suppressor (Ts) cells generate suppression of IgG antibody production to epitopes such as DNP presented on carrier molecules in carrier/hapten-carrier immunized animals (Herzenberg and Tokuhisa, 1982). Following this, Sun and Waltenbaugh (1986) demonstrated that the effecter cells of epitopic suppression are the $CD8^+$ T cells. Even after carrier/hapten-carrier immunization, subsequent anti-hapten responses remain suppressed even when the following immunization is with the hapten linked to an unrelated carrier. This would suggest the presence of an epitope-specific suppressor population and indeed, *in vitro* studies have indicated that the effecter cells responsible for the suppression are T cells with either some (Tagawa et al., 1984) or most of them (Schutz et al., 1987) bearing the surface CD8 antigen. Heal et al. (2001) indicated that adjuvant is also extremely important to elicit the appropriate and effective immune response against the antigen and they showed that blockade of the development of CD8+ T cells abrogates protection.

In 1989, Schutze et al. proposed a two-step mechanism for epitopic suppression. First, is a carrier specific, but not hapten specific, effect that results from clonal dominance after the expansion of the clones specific for the epitopes of the priming carrier. Second, after administration of the hapten-carrier conjugate to the already primed

animal, there is epitopic suppression that is mediated by Ts cells which is hapten specific (Schutze et al., 1989). These authors concluded that epitopic suppression is induced through the expansion of clones specific for the carrier epitope and that there is antigenic competition between the carrier epitopes the hapten.

Shortly after this proposal, Leclerc et al. (1990) proposed an alternative to the common belief that $CD8^+$ T cells were the only effecter cells involved in epitopic suppression. It was found that $CD4^+$ T cells play a major role in the induction of suppression and that B cells could be the regulator cells of epitopic suppression (Leclerc et al., 1990). By depletion of $CD4^+$ T cells, induction of epitopic suppression was prevented *in vivo* in this study. In contrast to the belief up to this point, it was found that CD8+ cytotoxic/Ts cells were not involved in the carrier-induced immunosuppression. Epitopic suppression does not require carrier-primed CD4+ or CD8+ cells for its expression (Leclerc et al., 1990), supporting the hypothesis that carrier-specific B cells may be the regulatory elements involved with epitopic suppression.

It appears there are differences in the induction and maintenance of suppression according to individual Ig isotype (Renjifo et al., 1998). IgG2a, IgG2b, and IgG3, and to a lesser extent IgG1 responses are significantly suppressed in carrier-primed animal while IgM responses show no evidence of suppression (Herzenberg et al., 1982). In carrier-primed animals, it was suggested that, selective deficiency in the specific hapten IgG2 response, that IFN-γ producing cells (TH1 cells) may not be properly activated (Renjifo et al., 1998). These authors hypothesized that epitopic suppression may result from defective hapten presentation by carrier-specific B cells that would efficiently capture the hapten-carrier conjugate and favor the development of Th2 cells. By priming with

dendritic cells and giving daily injections of IL-12, the authors were able to prevent the epitopic specific suppression. It was also determined that the induction of an optimal Th-1-type response was not sufficient to bypass the suppression. It was therefore determined that epitopic suppression may involve a defective hapten presentation since targeting the conjugate on dendritic cells is required to prevent the suppression. In agreement with other authors (Leclerc et al., 1990), Miyashita et al. (1997) determined that activated B cells down-regulate the expression of CD40 ligand/CD154 by $CD4^+$ T cells.

Immunization using a hapten-carrier conjugate containing bacteria or bacterial derivatives have been shown to induce the maturation of dendritic cells *in vitro* and *in vivo* (Roake et al., 1995; De Smedt et al., 1996) as well as migration of dendritic cells to T cell areas (De Smedt et al., 1996). It is speculated that bacterial components favor the presentation of hapten-carrier by competent dendritic cells. This would decrease the probability of epitopic suppression since dendritic cells have been found to play a major role in decreasing this phenomena (Renjifo et al., 1998).

In 1991, Sad et al. studied the regulation of carrier-mediated immune suppression in a strain of mice that are found to be non-responders to a vaccination to LHRH. It was determined that when mice were immunized using an alternate carrier, mice produced high levels of anti-LHRH antibodies. It was suggested that one carrier was unable to induce appropriate helper T cells capable of helping B-cells in these mice. Another possibility is that immunization with one carrier/LHRH leads to the generation of suppressor cells. It was determined (Sad et al., 1991) that immunization with one conjugate resulted in the generation of suppressor cells. These results agree with those of

Herzenberg et al. (1983) who found that epitope-specific regulation is mediated by suppressor T-cells.

It does appear that carrier mediated immune suppression may be a problem in mice and sometimes humans when receiving multiple immunizations, all attached to the same carrier. No data in cattle have been reported indicating this to be a problem in that species. It also appears that the presence of a Ts cell is still questionable as is the exact mechanism behind the action of carrier mediated immune suppression.

The general hypothesis proposed in this research is that immunization with LHRH fusion proteins will stop cyclicity in cattle. More specifically, different combinations of fusion proteins will be evaluated for effectiveness at suppressing the estrous cycle. To test these hypotheses, one main objective will be examined. The objective is to evaluate different carrier/hormone vaccination schedules to determine which is the most effective at sterilizing beef heifers.

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Chapter II

LHRH FUSION PROTEIN VACCINES IN BEEF HEIFERS

ABSTRACT

Two LHRH fusion proteins, thioredoxin (TL) and ovalbumin (OL), each containing seven LHRH inserts were tested. Objectives were to evaluate immune and biological responses from alternating the two fusion proteins in an immunization schedule. One hundred and ten heifers were divided equally into 11 groups. Control groups were either spayed or intact non-treated animals. Heifers in the other nine groups were immunized on wk 0, 4 and 8. Treatments were immunizations of the same protein throughout or alternating the proteins in different booster sequences. Blood was collected weekly for 22 wk and serum assayed for progesterone and LHRH antibody binding. At slaughter, reproductive tracts were removed from each heifer and weighed. Heifers with progesterone ≥ 1 ng/ml were considered to have a functional corpus luteum and thus have estrous cycle activity. All LHRH immunized groups of heifers had lower ($P < 0.05$) numbers of cycling animals after wk 6 when compared to the intact non-treated control group. There was no difference in number of heifers cycling between the immunized groups and the spayed heifers during wk 9 to 22. Anti-LHRH antibodies did not differ among immunized groups during wk 1 to 9. Starting at wk 10 and continuing through the conclusion of the study, there was an overall difference among treatment groups for anti-LHRH ($P < 0.05$). Uterine weights differed between treatments ($P < 0.05$) with intact control animals having heavier uteri than all other groups ($P < 0.05$). Uterine weights were negatively correlated with maximum LHRH antibody binding (r = -.44). In summary, these LHRH fusion proteins were as effective as surgical spaying in

suppression of estrous cycle activity, but, alternating the two proteins in an immunization schedule did not enhance the immunological or biological effectiveness of the vaccine.

Key Words: Heifers, Immunization, LHRH, LHRH fusion protein

INTRODUCTION

Approximately 12 million heifers are fed in the feedlot each year; of these 7.3% are pregnant at the time they enter the feedlot (USDA, 2000). Immunization against LHRH before entering the feedlot could sterilize heifers and prevent unwanted pregnancies that could reduce feedlot performance. To date, most LHRH sterilization vaccines used in female cattle have been produced using chemical conjugation techniques. Exceptions to this include techniques reported by of van der Zee et al. (1995), Cook et al. (2000), and Sosa et al. (2000), who utilized recombinant DNA technology to produce LHRH vaccines as a fusion protein for use in cattle. In order for a vaccine to be approved by the FDA, both within batch and between batch variability must be minimized, something that can be accomplished with a fusion protein much more easily than using chemical conjugation. Schutze et al. (1985) defined carrier-mediated immune suppression as pre-immunization against a given carrier that will prevent the elicitation of an immune response to a new antigenic epitope when presented on the same carrier. Sad et al. (1991) reported carrier-mediated immune suppression that resulted in the inhibition of immune responses to a hapten linked to the same carrier used in the initial immunization. In the current study, two different fusion proteins were utilized in an attempt to counteract possible carrier-mediated immune suppression. The hypothesis

of this study is that by alternating the carriers in an LHRH immunization schedule, the number of non-responding animals will decrease.

MATERIALS AND METHODS

Preparation of Antigens and Immunization

Ovalbumin-LHRH-7 was prepared by recombinant DNA technology as described by Zhang et al. (1999). An ovalbumin gene fragment (amino acid (AA) 18 to 381) was manipulated with the insertion of two LHRH sequences in tandem at the C-terminus (AA 381), monomers between AA 65 and 66 and between 97 and 98, and three sequences in tandem at the N-terminus (AA 18) (Figure 1). Purification of ovalbumin-LHRH-7 was performed as described by Zhang et al. (1999). Thioredoxin-LHRH-7 was prepared by recombinant DNA technology as described by Quesnell et al., (2000). A thioredoxin gene was manipulated with the insertion of two sequences in tandem at the C-terminus (AA 124), two sequences in tandem between AA 34 and 35 and three sequences in tandem at the N-terminus (AA 1) (Figure 1). Thioredoxin-LHRH-7 was purified as described previously for ovalbumin-LHRH-7.

All plasmids were expressed in *E. coli* BL21(DE3) (ovalbumin) and *E. coli* AD494(DE3) (thioredoxin). Both ovalbumin-LHRH-7 and thioredoxin-LHRH-7 were found to be insoluble and were thus dissolved in 6 M urea, centrifuged, and filtered through a .45-µM membrane prior to purification. These recombinant proteins contain six histidines (His-Tag; Novagen) on the C-terminal end which bind to heavy metals such as Ni^{2+}. To purify the protein, metal chelation affinity chromatography was conducted on

a Ni^{2+} column. The bound, purified protein was then eluted off the Ni^{2+} column with buffer. An SDS-Page gel was run with the purified protein to determine whether a product of correct size was present.

Primary immunizations were performed in a water-oil emulsion (containing *M. butyricum* as the immunostimulant) with 0.4 nmol of the LHRH antigen. Both booster immunizations were administered in a water-oil adjuvant without *M. butyricum* with an equal molar amount of LHRH antigen.

Animals and Treatments

All procedures performed on heifers in this study were approved by the Washington State University Institutional Animal Care and Use Committee (#1780). One hundred ten heifers at an average weight of 355 ± 3 kg were purchased from a local sale yard. Prior to the beginning of the study, blood was collected two times 14 d apart to determine serum progesterone concentrations. Palpation per rectum and the serum progesterone concentrations were used to determine estrous cycle activity and pregnancy in all heifers. Heifers were then divided into two groups, those that were cycling prior to the beginning of the study, and those that were not. Three consecutive wk of serum progesterone less than 1 ng/ml at any time during the study was used to indicate the heifer was acyclic at that time. Each group of heifers was then randomly and equally divided into the 11 treatment groups. Treatment groups were administered a primary immunization dose followed by two booster injections at 1 mo intervals. Immunizations were for either ovalbumin-LHRH-7, thioredoxin-LHRH-7 or their combination as described in Table 1, with the combination immunization receiving 0.2 nmol of each

fusion protein. Immunizations were given in one s.c. site on the neck of the animals. Ovariectomies were performed in 10 heifers using a transvaginal approach (Garber et al., 1990) before the start of the study to produce the spayed control heifers. This treatment group contained both cycling and non-cycling heifers before spaying.

Heifers were housed in 14 pens and fed a feedlot diet throughout the course of the study. Heifers were placed in pens based upon their weight so feed offered per pen could be adjusted accordingly.

Data Collection and Blood Samples

Blood samples were collected by coccygeal venipuncture to determine serum progesterone and LH concentrations as well as antibodies against LHRH, ovalbumin and thioredoxin. Serum samples were collected weekly for each of 22 wk. Serum progesterone analysis was conducted for all 22 wk, while analysis for serum LH, anti-LHRH, anti-ovalbumin and anti-thioredoxin antibodies were performed on samples collected every wk until wk 4 and then every other wk for the remainder of the study. Heifers were weighed every 28 d and reproductive tracts were removed and weighed at the time of slaughter. Uteri were trimmed of any visible fat and cut at the cervical/uterine junction before being weighed. Heifers were sent to slaughter when it was visually estimated that 70% would grade choice. Serum progesterone concentrations were utilized to determine estrous cycle activity. Three consecutive weeks of serum progesterone less than 1 ng/ml at any time during the study was used to indicate the heifer was acyclic at that time.

Iodination of Thioredoxin

One Iodo-bead (Pierce, Rockford, Il) was washed with 1.0 ml of 0.1 M $NaPO_4$ buffer (pH 6.5), dried on filter paper and placed into a 1.5 ml microcentrifuge tube. One hundred µl of 0.1M $NaPO_4$ (pH 6.5) was added to the microcentrifuge tube along with 10 µl [$Na^{125}I$] (1 mci) and allowed to react for 5 min. Five micrograms thioredoxin (Sigma, Saint Louis, Mo) in 35 µl 0.1 M $NaPO_4$ (pH 6.5) were then added to the tube and allowed to react for 8 min. The reaction was stopped by transferring the solution to a 3-ml column of anion exchange resin and eluted with 0.05 M PO_4 buffer until approximately 4 ml of elution are collected.

Hormone and Antibody Concentrations

Progesterone concentrations were quantified by a single-antibody RIA using a [^{125}I] progesterone Coat-a-Count kit (DPC, Los Angeles, CA.). Luteinizing hormone concentrations were quantified by double-antibody RIA (Adams et al., 1975). Percentage of [^{125}I] LHRH bound for each sample was quantified at a 1:1000 dilution with the [^{125}I] LHRH binding assay described by Johnson et al. (1988). Percentage of [^{125}I] thioredoxin bound for each sample was quantified by an RIA binding assay. The sera were diluted 1:1000 in EDTA-PBS, pH 7.0. Two hundred microliter of each diluted serum was added to assay tubes containing 400 µl PBS-gel, pH 7.0 (0.1% gelatin), and 200 µl normal bovine serum diluted 1:400 in EDTA-PBS. Sera were incubated for 24 h at 4°C with 30,000 cpm iodinated thioredoxin in 100 µl PBS-gel. Following the 24-h incubation, 200 µl of sheep anti-bovine gamma globulin diluted 1:15 in PBS, pH 7.0 was added as a second antibody to precipitate the antibody-bound labeled hormone. Assay tubes were

again incubated at 4°C for 24 h, followed by addition of 1 ml of PBS (pH 7.0). Tubes were centrifuged at 1500 x g for 20 min, the supernatant was poured off, and the pellet was counted in a gamma counter for bound radioactivity. The ELISA assay described by Zhang et al. (1999) was used to determine ovalbumin antibody concentration at a 1:10,000 dilution.

Statistical Analysis

Antibody titers for LHRH, thioredoxin and ovalbumin were initially analyzed with repeated measures analysis of variance (SAS Inst. Inc., Cary, NC). Due to interactions between treatments and time these data were analyzed at each time period using a completely randomized design. The model contained 11 treatments, cycling status of heifers before the beginning of the study and the interaction of the two as well as heifer BW as a covariate. The interaction was not significant at any of the time periods and were therefore removed from the test. When overall F-ratios were significant, differences among treatments were tested using pre-planned, non-orthogonal contrasts at the 5% probability level. A *post hoc* test was run to determine whether treatment groups containing two or more OL immunizations differed from treatment groups containing two or more TL immunizations. Average concentrations of LH, ADG, and uterine weight were analyzed using a one-way (11 treatments) ANOVA in a completely randomized design. A protected Waller-Duncan test statistic (SAS Inst. Inc., Cary, NC) at the 5% probability level was used to compare treatments. There were no differences among treatment groups for ADG or LH; therefore, treatment groups were pooled and compared to spayed and non treated heifers. Average daily gain and LH were also analyzed with a

repeated measures ANOVA. The model contained treatment, time and the treatment by time interaction. Concentrations of progesterone measured each wk were analyzed separately by one-way ANOVA using a completely randomized design with 11 treatments. When overall F-ratios were significant, differences were tested using pre-planned, non-orthogonal contrasts at the 5% level of probability. Pearson product moment coefficients of correlation were determined among mean and maximal LHRH antibody titers, uterine weight, ADG, start BW and final BW for all heifers (n=110).

RESULTS

Number of heifers cycling

The number of heifers cycling in each group did not differ ($P = 0.72$) at the beginning of the study. Beginning the second wk of the study, differences did exist between the non-treated control, spay control and treated groups ($P < 0.05$). All immunized groups of heifers had fewer cycling heifers after wk 6 when compared to the intact non-treated control group (Figure 3). During all 22 wk of the study, there were no overall differences in number of heifers cycling among immunized treatment groups ($P > 0.05$). A *post hoc* test to determine whether treatment groups containing two or more OL immunizations differed from those containing two or more TL immunizations indicated that from wk 1 to 8 and 13 to 16 there were no differences ($P > 0.05$). However, during wk 9-12 and 17-22, the number of heifers cycling differed ($P < 0.05$), with treatment groups containing two or more OL immunizations having fewer animals cycling during this time (Figure 2).

Antibody Titers

All animals actively immunized against the LHRH fusion proteins (90 of 90) developed measurable anti-LHRH antibody titers by the sixth wk of the study (Figure 3). As expected, measurable antibody titers were not detected in either the spayed control or non-treated control heifers during any of the 22-wk of the study (Figure 3). Pre-treatment cycling status did not influence ($P > 0.05$) anti-LHRH antibody titers during any of the 22 wk of the study. Beginning on wk 10 and continuing through the conclusion of the study, anti-LHRH antibody titers differed among the treatment groups. During wk 10, the OLOLOL group had higher ($P = 0.03$) anti-LHRH antibody titers than the TLTLTL group. Neither OLOLOL nor TLTLTL differed ($P > 0.05$) from the cocktail group. During wk 14, the OLOLOL and cocktail groups were not different from one another ($P = 0.74$) while both groups had higher ($P < 0.05$) anti-LHRH antibody titers than the TLTLTL groups. A similar trend was noted for wk 18 and 22. Antibody titers to LHRH were highest ($P < 0.05$) in the OLOLOL and cocktail heifers and lowest in the TLTLTL heifers.

Antibody titers for thioredoxin as indicated by RIA did not differ ($P > 0.05$) during the first 3 wk of the study. Beginning at wk 4 and until the conclusion of the study, percent binding differed among treatment groups ($P < 0.05$) (Figure 3).

Antibody titers for ovalbumin as indicated by ELISA did not differ during the first wk of the study ($P > 0.05$), however, from wk 2 through the conclusion of the study, ovalbumin antibody titers as expressed by optical density differed ($P < 0.05$) among treatment groups (Figure 3).

Average circulating concentrations (ng/ml) of LH during the study differed by treatment group ($P < 0.01$) with spayed control heifers having higher concentrations of LH than all other treatment groups (Table 2). When pooling all immunized groups and comparing them to the intact control and spayed control groups it was determined that the spayed control animals had higher ($P < 0.01$) circulating concentrations of LH while there was a trend for the immunized heifers to have a lower circulating concentration ($P = 0.06$) of LH than the intact control heifers (Table 2). Repeated measures analysis indicated a difference ($P < 0.05$) among treatment groups for concentration of LH but there were no time or treatment by time interactions between treatment groups ($P > 0.05$).

Average Daily Gain

Heifer BW were not significantly different between treatment groups at the beginning of the study ($P > 0.05$). Average daily gain did not differ among treatment groups ($P = 0.20$) from wk 4 through the conclusion of the study. However, pooling all immunized groups and comparing them to the intact control and the spayed control groups indicated that the immunized heifers had a lower ($P < 0.01$) ADG than the intact control heifers (1.0 ± 0.2 vs 1.2 ± 0.2 kg/d respectively) and were not different ($P > 0.05$) from the spayed control heifers (1.0 ± 0.2 vs 1.0 ± 0.2 kg/d respectively) (Table 2).

Uterine Weight

Uterine weight among treatment groups differed ($P < 0.01$) with the intact control heifers having heavier uteri (210 ± 6 g) than all other treatment groups (Table 2). The

spayed and immunized heifers had similar uterine weights (80 ± 13 and 90 ± 4 respectively) ($P > 0.05$) with the exception of the TLTLTL and OLOLOL groups. The TLTLTL groups had a heavier uterine weight than the OLOLOL group (106 ± 15 and 70 ± 5 respectively) ($P = 0.05$).

Correlation Coefficients Between Traits

There was a positive and high correlation ($P < .0001$) (r = .95) between mean LHRH antibody titer and high LHRH antibody titer. There was a negative correlation ($P < 0.05$) between high LHRH antibody titer and uterine weight and a positive correlation ($P < 0.05$) between starting BW and final BW, uterine weight and final BW, and ADG and final BW. There was no correlation ($P > 0.05$) between ADG and starting BW, antibody titer (mean or maximum) and either starting BW, final BW, or ADG (Table 3).

Carrier-Mediated Immune Suppression

Carrier-mediated immune suppression may be defined as an inhibition to the hapten portion of a vaccine after repeated immunizations with the same carrier protein (Sad et al., 1991). As can be seen in Figure 3, after each immunization, antibody titers increased ($P < 0.05$) for both the carrier protein and LHRH (the hapten portion of the vaccine). Thus, no carrier mediated immune suppression was observed in this study.

Table 1. Experimental design to evaluate combinations of ovalbumin-LHRH (OL) and thioredoxin-LHRH (TL) immunization schedules in heifers

Treatment Group	Primary Immunization	1st Booster Wk 4	2nd Booster Wk 9
1	Non-treated Control		
2	Spayed Control		
3	TL	TL	TL
4	TL	OL	TL
5	TL	TL	OL
6	TL	OL	OL
7	OL	OL	OL
8	OL	TL	OL
9	OL	OL	TL
10	OL	TL	TL
11	OL+TL	OL+TL	OL+TL

Table 2. Mean serum concentrations (ng/ml) of LH, uterine wt. and ADG during the 22-wk study for intact control, spayed control and pooled immunized treatment heifers. Mean ± SEM.

Treatment	LH (ng/ml)	Uterine wt. g	ADG, kg
Intact n=10	0.3 ± 0.1^{a}	210 ± 6^{a}	1.2 ± 0.1^{a}
Spayed n=10	0.9 ± 0.1^{b}	80 ± 13^{b}	1.0 ± 0.1^{b}
Immunized n=89	0.1 ± 0.1^{a}	90 ± 4^{b}	1.0 ± 0.1^{b}

[a,b]Values with different superscripts within the same column differ, $P < 0.05$

Table 3. Correlation coefficients between different traits of all immunized beef heifers

Item	ADG	Final BW	Start BW	Uterine wt.	Max ab titer
Mean ab titer	-.10	-.08	-.01	-.42*	.95*
Max ab titer	-.06	-.02	.03	-.44*	
Uterine wt.	.29*	.22*	.03		
Start BW	.15	.73*			
Final BW	.78*				

*$P < 0.05$.

Figure 1. Schematic structures of recombinant ovalbumin, ovalbumin-LHRH-7 ($MW_C \approx$ 55KDa), thioredoxin and thioredoxin-LHRH-7 (MW ≈ 22 KDa). The straight lines represent amino acids (aa) (18-381 for ovalbumin and entire sequence for thioredoxin). Open boxes represent LHRH insertion sites for ovalbumin (before aa 18, between aa 65 and 66, between aa 97 and 98, and after aa 381) and thioredoxin (before aa 1, between aa 34 and 35, and after aa 124). The closed box at the C-terminus represents the His-tag sequence used for His-bind affinity chromatography

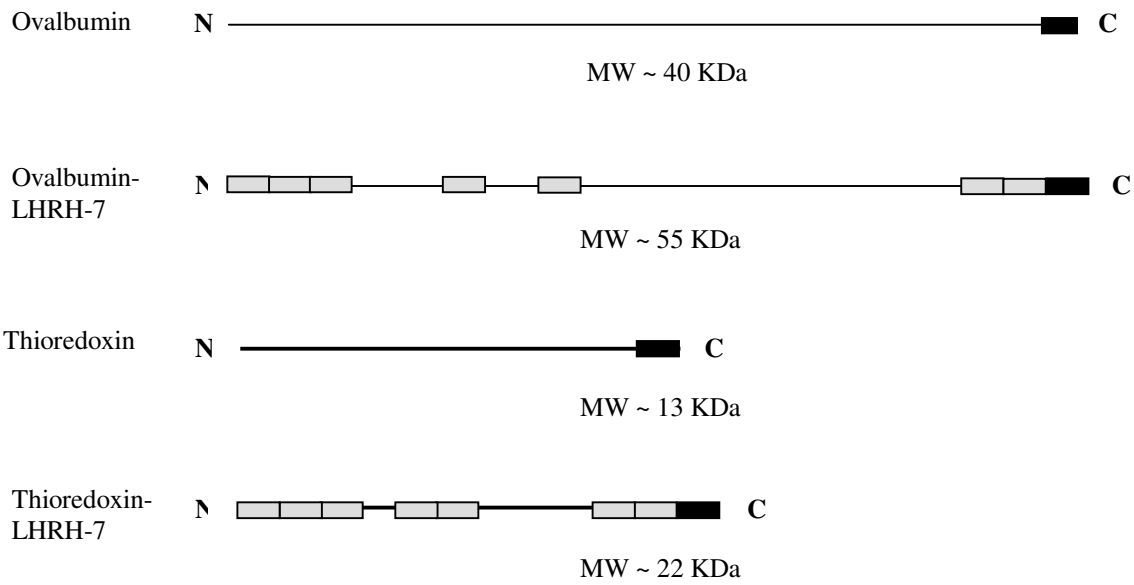

Ovalbumin
N
C
MW ~ 40 KDa
Ovalbumin-
LHRH-7
N
C
MW ~ 55 KDa
Thioredoxin
N
C
MW ~ 13 KDa
Thioredoxin-
LHRH-7
N
C
MW ~ 22 KDa

Figure 2. Percent of heifers cycling (as determined by weekly serum concentrations of progesterone) in groups with two or more TL immunizations (▲) (n=40) two or more OL immunizations (■) (n=39), and in intact control heifers (●) (n=10). Arrows indicate time of immunizations. Treatment differences existed between OL and TL groups from wk 9-12 and 17-22 ($P < 0.05$) and percent of heifers cycling in both TL and OL groups were reduced ($P < 0.05$) compared to intact control heifers by wk 6.

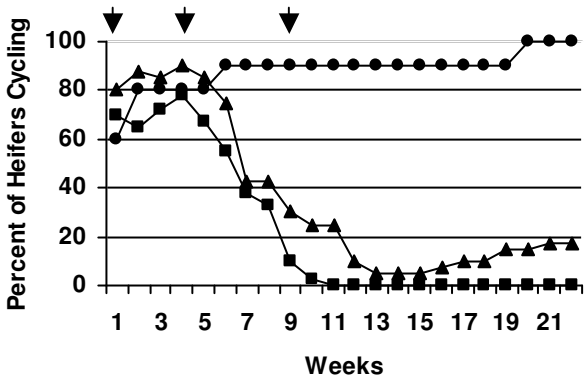
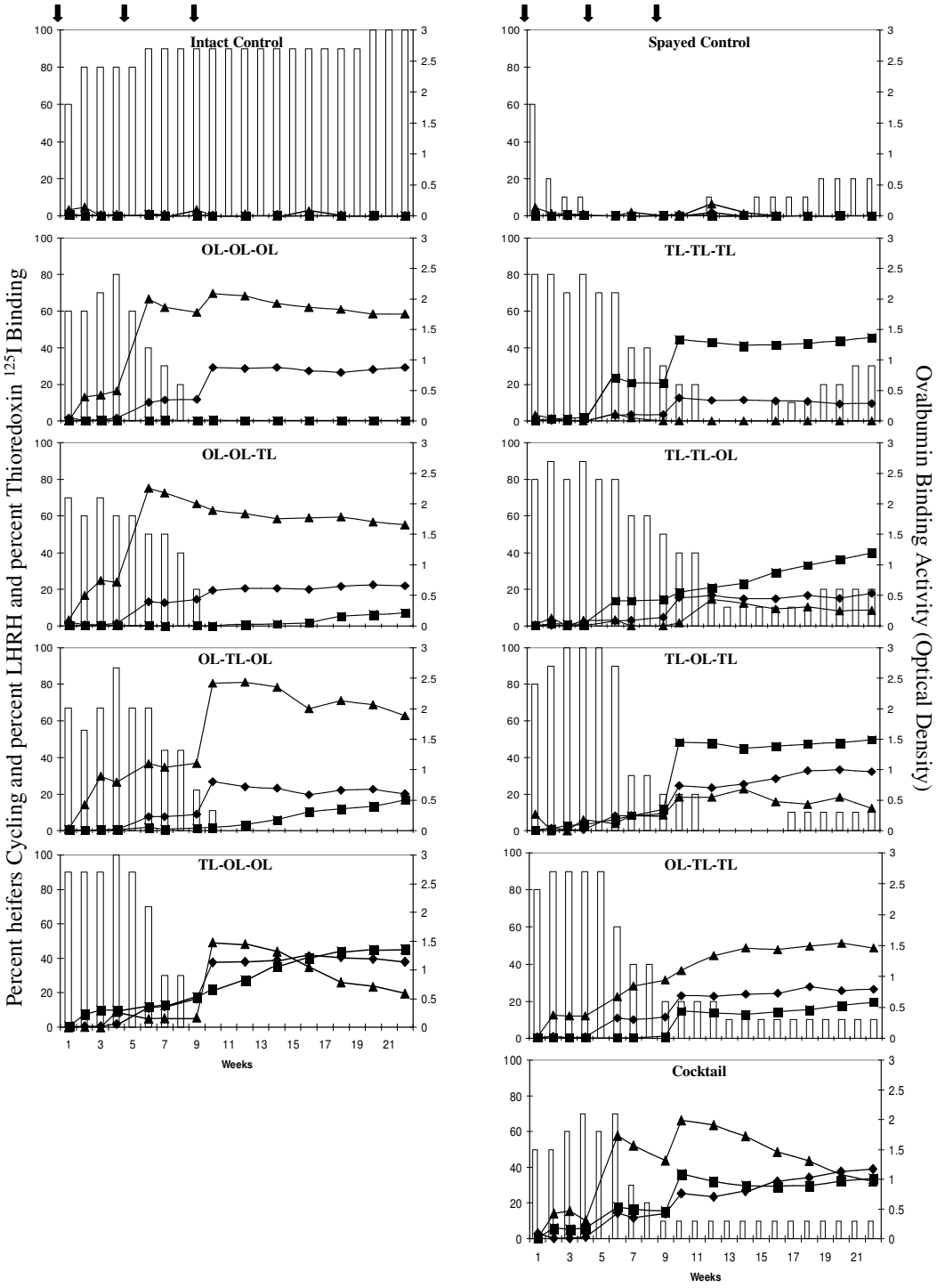

Percent of Heifers Cycling
100
80
60
40
20
0
1
3
5
7
9
11
13
15
17
19
21
Weeks

Figure 3. Percent of heifers cycling (bars) and percent LHRH (♦) and thioredoxin (■) ^{125}I binding (left axis) and ovalbumin (▲) antibody titers (right axis) after LHRH immunization using different carriers. Arrows indicate immunization times. Anti-LHRH antibody titers were elevated ($P < 0.05$) in all immunized groups of heifers by wk 6 and there were fewer ($P < 0.05$) cycling heifers at this time when compared to intact non-treated heifers.


Intact Control
Spayed Control
OL-OL-OL
TL-TL-TL
OL-OL-TL
TL-TL-OL
OL-TL-OL
TL-OL-TL
TL-OL-OL
OL-TL-TL
Cocktail
Weeks
Weeks
Percent heifers Cycling and percent LHRH and percent Thioredoxin ^{125}I Binding
Ovalbumin Binding Activity (Optical Density)

DISCUSSION

Immunoneutralization of LHRH disrupts the endocrine cascade that allows for follicular growth and ovulation, ovarian steroidogenesis and displays of behavioral estrus (Adams and Adams, 1986). Thus, serum LH concentrations are lowered when heifers are immunized against LHRH and the uterus of immunized heifers reverts back to a more regressed state. Adams and Adams (1990) showed that pituitary concentrations of both LH and LHRH receptors are decreased by approximately one-half in immunized animals vs. their non-immunized contemporaries. In the current study, this disruption of the endocrine cascade is readily apparent after the seventh wk of the study when there was a decrease in the number of cycling heifers. This trend of decreased numbers of cycling heifers continued until week 13 when the number of cycling heifers in all treated groups was 3 of 89 animals. Thus at this time, only 3% of the treated animals were cycling compared to 90% of the intact control heifers.

Evidence of a carrier-mediated immune suppression as suggested by Sad et al. (1991) was not evident in this study. Treatment groups receiving each immunization with the same carrier had an increase in anti-LHRH antibody titers, similar to what was seen when alternating carrier proteins. This is also evident by the lack of differences in the number of cycling animals in each treatment group throughout the study. Luteinizing hormone-releasing hormone antibody titers were significantly elevated in all treatment groups when compared to the intact control and spayed control heifers by the 10th wk of the study.

Serum concentrations of LH were as expected for the different treatment groups. Spayed control heifers had the highest serum concentration of LH when taken as an

average throughout the study, which is consistent with previous observations after castration in cattle (Kiser et al., 1981).

Average daily gains did not differ among treatment groups which is similar to that reported by Prendiville et al. (1995) and different from that of Adams and Adams (1990) when heifers did not receive an anabolic steroid implant. A more relevant comparison can be made when all immunized animals are pooled and compared to the intact control and spayed control heifers. This approach revealed that the intact control group had a greater ADG than both the immunized and spayed control groups as would be expected for animals with an endogenous source of estrogens (Lammers et al., 1999). The spayed control groups did not differ from the immunized groups as would be expected due to large decreases in circulating estrogens. Adams et al. (1990) showed that Synovex H implants allow for BW gains in spayed and LHRH-immunized heifers that are similar to that of intact heifers. From a production standpoint, this lack of endogenous steroid production while animals are being treated with an LHRH vaccine will negatively affect growth. However, it is important to note that the objectives for this vaccine are to block estrous cycling and pregnancy, much the same as spaying is used on range heifers prior to entering the feedlot in the Western United States. Immunization is not being proposed as a replacement for use of melengestrol acetate in the feedlot. Once LHRH-immunized heifers enter the feedlot, it will be necessary to utilize growth promotants, similar to that for spayed heifers, to overcome the lack of ovarian steroid production. There are other options to a LHRH vaccine that may not have the same effect on growth. For instance, a conjugated porcine zona pellucida vaccine has been developed for horses and deer

(Kirkpatrick et al., 1997) that still allows for normal estrous cycle activity while blocking sperm binding to ovulated ova.

One interesting observation was made when assessing antibody titers for a given carrier protein. When boosting with one carrier, we continue to observe an increase in antibody titer to the opposite carrier. This would indicate an increase in the antibody production of the carrier not being boosted for. For this to occur, both fusion proteins must have an epitope in common. The C-terminal His-tag, which is common to both fusion proteins, may cross-react when producing antibodies. This trend in boosting antibody production of the carrier was seen for both carrier proteins and may also be a reason that no carrier-mediated immune suppression was seen in this study (Figure 3).

Figure 3 indicates that two heifers in the spayed control group resumed cycling before the end of the study. This is most likely due to a small amount of ovarian tissue having been left following ovariectomy, which could have allowed a small amount of luteal tissue to develop. Similar results have been reported by Garber et al. (1990) when using a similar procedure for spaying heifers. While heifers in their study did not have discernable ovaries at the time of slaughter, enough tissue apparently remained allowing for progesterone concentrations to be elevated relative to those observed in non-cycling heifers. While it is possible that other sources of progesterone also contributed to the elevated concentrations of progesterone (adrenal progesterone), serum progesterone in immunized heifers, which should have exhibited similar patterns remained depressed. The fact that some heifers resumed estrous cycling before the conclusion of the study lends credence to idea that the effects of the vaccine are reversible. Although these heifers were not subjected to a bull for breeding and their ability to become pregnant was

not tested, resumption of cyclicity is a strong indication that effects of the vaccine may be reversible in some heifers. Bishop et al. (1996) found that LHRH-immunized heifers could be induced to ovulate with gonadotropins, also supporting the idea that heifers are able to undergo folliculogenesis and subsequent ovulation with proper stimuli. Since no animals immunized with two or more OL immunizations resumed cycling, and the study was concluded after 22 wk, it was impossible to determine whether reversibility of the vaccine in these treatment groups would occur. By the conclusion of the study, 17.5% (7 of 40 animals) of the animals treated with two or more TL immunizations were cycling as determined by serum concentrations of progesterone. Of these seven heifers, three were in the TLTLTL treatment group. A difference in the effectiveness of the two fusion protein LHRH vaccines was obvious in this study. It is quite possible that protein folding may hide several of the LHRH inserts in the TL fusion protein, thus making this protein less effective in producing antibodies against LHRH.

IMPLICATIONS

The effectiveness of the LHRH fusion protein vaccine was 97% in all treated groups by wk 13, therefore lending strong support to the ability of the ovalbumin-LHRH fusion protein vaccine to perform as expected and effectively sterilize heifers prior to their entering the feedlot. The presence of a carrier-mediated immune suppression in heifers immunized with the same carrier protein each of 3 times was not found to exist in the current study.

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Chapter III

REVIEW OF LITERATURE

INTRODUCTION

Preantral follicular development

Follicles form when primary oocytes are enveloped by a single layer of flattened granulosa cells (GC's) (Fortune, 2003). Synchrony of follicle formation is species specific with it occurring fairly synchronous in mice but not very synchronous in most domestic animals or primates. The time of follicle formation also differs among species with formation occurring shortly after birth in mice but occurring during fetal life in most domestic species (Fortune, 2003). During the time of envelopment of oocytes with this layer of flattened GC's, the follicles enter a resting pool that will be utilized throughout an animals reproductive life. During each reproductive cycle, a cohort of follicles is selected from this resting pool, the GC layer becomes cuboidal and begins to express markers of cell proliferation (Fortune, 2003). In the mouse follicle, this single layer of GC's increases in cell number until approximately 60 GC's are present. After this time, a second layer of GC's develops, followed by several more layers until 4-6 layers of GC's are present. The follicle is characterized as a secondary follicle during this time. During the late secondary follicle stage (4-6 layers of GC's), between 200 and 400 GC's are present in the mouse follicle (Pedersen and Peters, 1968; Lintern-Moore and Moore, 1979). Termination of the secondary follicle culminates with the gradual development of an antral cavity. At this time, stromal cells are recruited to form the thecal layer (Fortune, 2003). In rodent follicles, the thecal layer can be present as early as the late

primary or early secondary stage (Fortune and Eppig, 1979), however in bovine, ovine and primate follicles it isn't until the mid to late preantral stage that thecal cells can be found (Gougeon, 1996; Braw-Tal and Yossefi, 1997; Lundy et al., 1999). Classification of follicles differs slightly from rodents to bovine, ovine and primates, therefore for ease, the stages will further be referred to as primordial, primary, small preantral, medium-sized preantral and large preantral.

Studying preantral follicular development

Several methods have been devised to study preantral follicular development in vitro. Rodent ovaries are small enough to be maintained in organ cultures and it is common to utilize whole ovaries of newborn rodents to study factors that may affect the entry of primordial follicles into the growing pool as well as the primary to secondary follicle transition (Fortune, 2003). In vitro, primordial follicles will commonly activate spontaneously and progress to the primary and possibly secondary stage in about 8 days in a fashion that appears to be temporally and qualitatively normal (Eppig and O'Brien, 1996). In contrast, in large mammals, the size of the ovary precludes their culture as intact units. Methods have been devised to culture small pieces or slices of the ovarian cortex, the area where the resting pool of follicles is located, thus allowing for the study of follicle activation and growth in cattle (Braw-Tal and Yossefi, 1997), primates (Wandju et al., 1997), and women (Hovatta et a., 1997). Unlike rodent ovaries however, when culturing cortical pieces of large mammals, there is almost complete primordial follicle activation and the follicles progress in masse to the primary stage where they can be maintained for up to 20 days (Wandji et al., 1997). Because of this almost total

convergence of primordial to primary follicle development, in vitro culture of large mammalian ovarian tissue is more suited to the study of factors that inhibit follicle activation than it is to those factors promoting follicle activation. Very few follicles that have made the primordial to primary transition are capable of progressing to the secondary follicle stage thus not allowing for the study of transition in vitro between these two stages.

More recently, ovarian cortical pieces from mice (Cushman et al., 2001) and cattle (Cushman et al., 2002) have been maintained "in ovo". In this type of culture, ovarian cortical pieces are grafted beneath the chorioallantoic membrane of chick embryos. Vascularization can occur in this situation and primordial follicle activation is almost completely absent (Cushman et al., 2001), thus allowing for the study of potential stimulators of follicle activation. Another method of culture has also been utilized in rodents (Callejo et al., 2002; Sato et al., 2003; Snow et al., 2002; Van den Broecke et al., 2000) and humans (Callejo et al., 2001; Imthurn et al., 2000; Oktay et al., 2003) in which cortical pieces of tissue are grafted under the skin (Callejo et al., 2001; Callejo et al., 2002; Imthurn et al., 2000; Oktay et al., 2003; Sato et al., 2003) or kidney capsule (Snow et al., 2002; Van den Broecke et al., 2000) of either rodents or humans.

Culture of secondary ovarian follicles is also more easily accomplished in rodents when compared to ruminants and primates (Fortune et al., 2003). Rodents have a soft stroma that is easily disrupted yielding follicles that survive in culture whereas ruminant and primate ovarian stroma is very dense and difficult to disrupt. Oocytes from ruminant and primates are more easily damaged when using enzymatic digestion (Wandji et al., 1996).

Control of Follicular Development

It is well established that antral follicle development is regulated by the gonadotropins but there is question as to the regulatory role of gonadotropins in the preantral follicle. Dufour et al. (1979) provided evidence suggesting that gonadotropins affect the development of follicles at the preantral stage. This was accomplished through hypophysectomy of ewes and finding large quantitative changes in follicular development. Similar results have been found in the mouse (Edwards et al., 1977). In rodents it has been determined that FSH and LH can facilitate the development of preantral follicles in vitro when the follicles are at least at an early secondary stage (Fortune and Eppig, 1979). It has also been determined that there is an FSH-independent phase in which addition of FSH to a cultured ovary will have no effect on follicular growth and differentiation (Liu et al., 1998, 1999). If ovarian tissue is cultured long enough however, it can enter into an FSH-dependent phase (Hartshorne et al., 1994). It has been shown in human ovarian cortical strips that culture with FSH on the smallest growing preantral follicles causes a decrease in the percentage of atretic follicles during the culture period (Wright et al., 1999). This finding is consistent with the detection of mRNA for the FSH receptor in human primary follicles in vivo (Oktay et al., 1997a). In contrast, culture of bovine cortical tissue with FSH does not appear to alter the number of atretic follicles during either short (Braw-Tal and Yossefi, 1997) or long (Fortune et al., 1998) cultures. There does appear to be receptors for FSH on GC's of bovine primary follicles (Tisdall et al., 1995) but there is also evidence that these receptors may not be coupled to adenylyl cyclase (Fortune et al., 1999).

Much less is known about the effects of LH on growth and development of preantral follicles. In mice it has been shown that LH is needed for development in vitro of smaller preantral follicles to the antral stage (Wu et al., 2000). It appears in cattle that selection of the dominant follicle from a cohort may be due to LH (Mihm et al., 2002). The first developing follicle that develops LH receptors is the follicle that continues to grow while the others regress due to a decrease in FSH secretion from the pituitary. This occurs in antral follicles however and the role of LH prior to this time is still in question. Other than the gonatotropins, there are many factors controlling follicular development at different stages. Some of these factors may act alone, or synergistically with other factors including the gonadotropins.

IGF and Insulin

Insulin has been shown to increase the percentage of healthy follicles when slices of human ovarian cortex are cultured (Louhio et al., 2000). Along with insulin, IGF-I and IGF-II additions to culture media will increase the number of follicles that are in the primary stage. In contrast to human ovarian tissue, the addition of IGF-I to bovine cortical pieces in culture has no effect on the proportion of primary and secondary follicles (Derrar et al., 2000). In contrast to Derrar et al. (2000), it was found that IGF-I had negative effects on follicular health, growth and activation when given in graded dosages (Yang and Fortune, 2002). It does appear that insulin at large doses (>500ng/ml) is required for follicular activation and subsequent survival of bovine follicles in vitro while IGF-1 interferes with the positive actions of insulin. Yu and Roy (1999) reported the effects of insulin on follicle development in cultured ovaries from hamsters to have a

biphasic effect with higher dosages being less effective than lower doses. In support of this finding is that of Kezele et al. (2002) who reported that insulin increased follicle activation in rat ovaries and these effects were additive with those of several other factors. There is no effect on follicular activation or early growth however when IGF-I is added to the media.

KIT/Kit ligand

There is evidence for the presence of both KIT and Kit ligand (KL) in rodents and sheep throughout gonadal formation and follicular development except during the early phase of meiosis I, prior to the formation of follicles. Kit ligand is expressed in two ways, a soluble form (KL-1) and a membrane-bound form (KL-2). In 1993, Manova et al. was able to detect mRNA for both forms of KL in both juvenile and adult mouse ovaries. Low levels of expression of the mRNA in GC's of small preantral follicles of mice and higher levels in larger preantral follicles have been found. Messenger RNA expression appears to differ slightly for KL in sheep ovaries. Tisdall et al. (1997) found KL expression in GC's of all follicles in sheep including primordial follicles. KIT mRNA expression has been localized in oocytes of primordial, growing preantral and antral follicles in both mice and sheep (Manova et al., 1990; Clark et al., 1996).

There is evidence supporting the role for KIT and KL interactions in both preantral follicular development and the initiation of follicular growth. For instance, if rat ovaries are treated with KL in vitro, the percentage of follicles in the growing pool increases relative to control ovaries (Parott and Skinner, 1999). This suggests a role for KL in the activation of primordial follicles. In bovine ovarian cortical tissue cultures,

almost all primordial follicles activate spontaneously and the addition of KL to these cultures does not appear to affect follicle populations.

While there are other growth factors that appear to, or do, regulate follicle development and activation at early stages of growth, the scope of this paper is not to cover every possible factor. Rather, the goal is to introduce some of the factors that have their function in regulation of follicular development somewhat elucidated. As can be determined, the actions of the presented factors appears to be different quite frequently in different species. This may make xenograft technology somewhat more challenging when grafting between species that have very different requirements for follicle activation and growth.

XENOGRAFT AND AUTOGRAFT STUDIES

Introduction

Cancer treatments involving radiation and chemotherapy are devastating to a young woman's reproductive lifespan. Recently, methods have been explored that can not only restore fertility to women that have undergone cancer therapy, but can also restore a normal hormonal profile for these women. Cryopreservation of ovarian cortical tissue followed by transplantation of this tissue back into the woman, has allowed for normal menstral cycles may be re-established. However, after cancer involving the reproductive system, it may be advisable to not transplant tissue that has the potential to contain cancer cells. Rather, grafting this tissue into an immunodeficient mouse or rat, allowing for follicular growth and then removing the oocyte from a competent antral

follicle for in vitro fertilization may still allow for children to be produced from a woman's genetics that would otherwise be impossible.

Other reasons for studying xenograft technology include the ability to study inhibitors or inducers for follicular development as well as the ability to study a rare problem through several different trials by transplanting cortical tissue from an afflicted animal to many recipient animals. For instance, in beef cattle that are superovulated frequently, some cows will quit ovulating. Even though these cattle have many oocytes left in their ovaries, the cattle are not responding to superovulation treatments any longer. Removing ovaries from these cattle and grafting pieces of the cortex into many different mice or rats will allow for different treatments to be applied to the mice in an attempt to cause folliculogenesis to occur. If the ovaries were left in the cow, very few attempts to induce follicular growth and ovulation would be possible.

Grafting ovarian tissue would also allow for the study of factors that activate follicular growth and differentiation in cattle. In ovarian tissue that is cultured, there is almost complete primordial follicle activation leading to almost all follicles entering the primary stage (Wandji et al., 1997). Placing this tissue into another animal model may allow for the study of factors that activate follicular growth as well.

Vascular development

Israely et al. (2003) indicated one of the most important factors for successful ovarian graft survival is the rapid establishment of a rich blood supply. This is required for the ultimate survival of the ovarian follicles in the graft (Weissman et al., 1999). Different results have been reported for follicle survival of mouse ovaries (Felicio et al.,

1983) and sheep ovarian tissue (Baird et al., 1999) grafts with a 50% and 35% survival of oocytes respectively. Kim et al. (2002) reported a reduction in graft size along with fibrotic changes of most grafts. Location of the graft may play a role in the rate of vascularization and thus may be a determining factor in the number of oocytes surviving the graft procedure. For instance, Israely et al. (2003) grafted tissue both subcutaneously and intramuscularly and determined vascular remodeling by MRI and histology. These authors determined that significant ovarian damage was observed in both cases within the first 24 h after transplantation. The intramuscular grafts appeared to recover in their study by becoming vascularized while the subcutaneous grafts remained impaired and necrotic. In contrast, Van den Broecke et al. (2001) reported that human ovarian grafts, when transplanted into SCID mice, were well vascularized in both subcutaneous and kidney capsule transplants. Similarly, Imthurn et al. (2000) found the addition of FSH treatment to increase the amount of revascularization in grafted ovarian tissue.

Grafted ovarian tissue in the rat showed a mix of viable ovarian tissue and ovarian tissue that were in varying stages of degeneration most likely brought about by ischemia (Corleta et al., 1998). These authors determined that slicing the rat ovary into 4 sections prior to grafting allowed for a better blood supply to be established than grafting whole ovaries. Animals receiving grafted tissue were able to maintain normal hormone profiles when compared to both intact control and ovariectomized control rats (Corleta et al., 1998).

Cryopreserved vs. fresh tissue

Our ability to preserve spermatozoa through storage has been available for many years (Polge et al., 1949), however attempts at freezing mammalian oocytes has been problematic. Cryopreservation of ovarian cortical tissue may allow for better preservation of oocytes than freezing individual oocytes and will allow for more genetic material to be saved quickly versus embryo freezing. Kagabu and Umezu (2000) cryopreserved ovarian tissue from several mammalian species prior to performing xenografts and determined that cryopreservation was successful since healthy follicles up to the small antral stage were capable of surviving. These authors indicated that freezing and thawing do not damage the ovarian tissue to a large degree, rather more of the damage to the graft is due to the actual grafting procedure. Gook et al. (2001) directly compared fresh to cryopreserved ovarian tissue and determined that no difference existed in the ability of the graft to survive and develop between the two. In 1994, Gosden et al. grafted frozen ovarian cortical pieces in one side of sheep and fresh tissue into the other side as a control. After 4 months the first signs of ovulation were detected. Following this, Baird et al. (1999) performed autotransplants with frozen tissue into eight sheep and followed these animals for 22 weeks. All of the animals resumed cyclicity and showed hormone production that was somewhat normal. Follicle stimulating hormone levels were elevated over normal animals most likely due to low serum inhibin-A levels.

Location of graft

Kagabu and Umezu (2000) found the uterus of recipient animals to be an extremely hospitable environment for grafted ovarian tissue. In 2004, Lee et al. grafted

ovarian cortical strips from monkeys into either the forearm, abdomen or under the kidney capsule of recipient monkeys. These authors found that recovery of oocytes from grafts under the kidney capsule was impossible while those grafts in the forearm and abdomen were easy to recover and made visualization of the grafts easy. Callejo et al. (1999) found no difference in intraperitoneal versus subcutaneous grafts in either longevity of the graft or of function.

Follicular survival

Van den Broecke et al. (2001) found a small shift in the proportion of primordial follicles after transplantation (74 to 68%) as well as an increase in the number of primary follicles (23 to 26%) and secondary follicles (2 to 5%). Similar results were seen with cryopreserved tissue in the same study. The study by these authors was the first to demonstrate that primordial follicles can grow from frozen-thawed human ovarian cortical grafts as well as those primordial follicles from fresh ovarian grafts when transplanted to immunodeficient mice. Another study (Nisolle et al., 2000) looked at the follicular density of fresh and frozen human ovarian cortical grafts at two sites. They found no difference existed in follicular density between frozen or fresh tissue or between location of the graft. However, it was noted that fresh ovarian biopsies contained more follicular density than any of the grafted tissue.

When autografting human ovarian tissue into the arm or rectus abdominus muscle of recipients, Callejo et al. (2001) found ovarian hormone secretion to be reestablished after 3-4 months and normal hormone secretion was maintained for an additional 2-4 months with the exception of inhibin levels which were undetectable. Even with such

positive results without exogenous hormone treatment it was determined that neither follicular dominance nor CL development were present on the grafted tissue. Similar results were found by Lee et al., 2004. Grafts into the forearm of monkeys resulted in follicular development that was monitored by ultrasound. When follicles reached 4-mm, monkeys were treated with 1,000 IU of human chorionic gonadotropin (hCG) and oocytes were collected 26 to 30 hours later. Plasma levels of E_2 and P_4 were higher in venous drainage of an arm receiving a graft than in systemic venous blood, indicating that grafted ovarian tissue appeared functional. One monkey had an abnormally long (roughly 60 days) luteal phase when compared to normal monkeys (Lee et al., 2004). These researchers indicated that the best follicular development was in abdominal subcutaneous grafts. Radford et al. (2001) found it took approximately 7 months for hot flashes to disappear in a woman that was autografted.

In 1986, Norman and Spies found, when grafting ovarian tissue into male Macaques, an increase in serum E_2 and a resulting increase in serum LH shortly after graft transplant. While not of the levels normally found in the female macaque, this evidence was important in determining that castrated male animals may be suitable recipient animals for ovarian tissue. Weissman et al. (1999) found no significant difference in the presence of primary or primordial follicles in male versus female mice as hosts of human ovarian grafts. Interestingly, after hormone stimulation there were signifgicantly more preantral follicles present in male recipients when compared to female recipients.

Xenografting common wombat ovarian tissue into nude rats was performed by Wolvekamp et al. (2001) as a test to determine whether this would be a possible means of

culturing banked ovarian tissue from the highly endangered Northern Hairy-nosed Wombat. Interestingly, these authors found ovarian tissue from one donor animal to become fibrotic in appearance and to contain no primordial follicles. Ovarian tissue from the other two donor animals was well vascularized however and contained many primordial follicles. One can assume that either the length of time from death to tissue collection (Wolvekamp et al., 2001) or a lack of vascularization was the cause of fibrosis and a lack of healthy follicles. Kim et al. (2002) found similar results, however, it was determined that 50% of grafts from an older donor became fibrotic and were devoid of follicles. Nisolle et al. (2000) determined that cryopreserved ovarian tissue contained more fibrosis after grafting than did tissue that was grafted fresh. Similar to other authors, Wolvekamp et al. (2001) did not find follicular development to the antral stage, however pre-antral follicles of all stages were found in grafted tissue. Other studies involving xenografting both marmoset and sheep ovarian tissue (Gosden et al., 1994) into SCID-mice were able to show antral stage development.

Viability studies have been performed on cryopreserved ovarian cortical pieces, specifically looking at the primordial follicle pool after the freezing process (Oktay et al., 1997b). These authors found that approximately 70% of follicles survived the freezing process and were viable.

Hormone treatment

Van den Broecke et al. (2001) found no follicular development beyond the two-layer stage in human ovarian transplants grafted into SCID mice with no hormone stimulation or in mice that were stimulated with gonadotrophin for 2 weeks after

transplantation. Identical results were found by Oktay et al. (2000). It has been determined (Gougeon, 1986) that follicular development to pre-ovulatory stages takes approximately 17 weeks and reports by Oktay et al. (1998) indicate that follicular development is halted at the two-layer stage even after 17 weeks in non-stimulated mice. Other researchers (Weissman et al., 1999) have been able to obtain antral growth of follicles after 12 weeks of transplantation following a short, 2 week, gonadotrophin stimulation. It is believed that grafted tissue has an FSH dependency in order to resume growth.

In human graft experiments using 0.1 mg of transdermal E_2 in an attempt to increase angiogenesis after the time of grafting, Oktay et al. (2003) found follicular development to take ten weeks in one patient and 5 months in another. Once follicular development occurred, E_2 was removed and normal menstral cycles were maintained for 21 months in one patient and 28 months in a second patient.

Production of live young

While the ability to restore hormone profiles in autografts and the ability to obtain follicular growth in xenografts has been proven in several studies, some question whether live young can be produced from xenografted tissue. Gosden et al. (1994) was the first to show that live offspring can be produced from either fresh or cryopreserved ovarian tissue when grafted to the infindibulopelvic ligament of sheep. Following this, Snow et al. (2002), utilizing mouse ovarian tissue that was halved and grafted under the kidney capsule of intact adult male and female nude rat recipients, indicated all male rats and some female rats were treated with PMSG and monitored for follicular development.

Xenografts were recovered and oocytes were removed from the grafted tissue, cultured for 18 hours and then inseminated with sperm and transferred to synchronized recipient mice. It was determined that female rats were a better recipient of the xenografts than male rats as significantly more ovarian xenografts were recovered from females. In total, 5 pups were born to females receiving embryos derived from the xenografts. All pups developed normally and were able to produce healthy live young themselves when they had reached sexual maturity. Following this, Lee et al. (2004) was able to produce a live monkey from ovarian cortical tissue that had been grafted subcutaneously in the abdomen of a female monkey.

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Chapter IV

BOVINE ECTOPIC OVARIAN XENOGRAFTING

ABSTRACT

Bovine ovarian tissue from two donor ages, fetal day 160 and adult, was grafted under the skin of nude mice. The objective was to determine if spontaneous differentiation of follicles occurs in grafted tissue. Thirty-two mice were equally divided to receive ovarian tissue from either adult or fetal day 160 ovaries. All recipient mice were ovariectomized prior to receiving between two and fours grafts from the same donor. Vaginal smears were performed daily to determine estrous cycle activity in grafted mice. Grafted tissue was removed from 1 to 24 wk later and analyzed for follicular development and the presence of FSH receptor. Follicular development did not get past the secondary stage in any of the xenografted tissue. Adult donor tissue appeared to develop fibrosis more readily than fetal day 160 tissue. The total number of follicles present in adult and fetal day 160 ovarian tissue favors fetal ovaries for the study of factors initiating follicular differentiation. The presence of follicle stimulating hormone receptor was only found in tissue from fetal day 160 ovaries that had been grafted for two weeks. In summary, spontaneous differentiation of follicles did not appear to occur in grafted ovarian tissue. Fetal day 160 tissue contained more total follicles and would thus be better for studying factors that initiate follicular development.

INTRODUCTION

Over the last 10 years, resurgence in the amount of interest related to ovarian explant research has occurred. Most of this interest is directed at finding ways to preserve ovarian tissue from young women with cancer. Not only will transplanting

ovarian tissue back into these women after cancer treatment has been completed allow for endogenous reproductive hormone production to occur (Radford et al., 2001; Oktay et al., 2003), it may allow for the production of offspring through an in vitro fertilization procedure (Snow et al., 2002; Lee et al., 2004). The function of grafted ovarian tissue, as assessed by both hormone secretion and follicular development, in both allografts and xenografts has been restored in a variety of animals including mice (Van den Broecke et al., 2001), rats (Snow et al., 2002), sheep (Baird et al., 1999), wallabies (Wolvekamp et al., 2001), monkeys (Lee et al., 2004) and humans (Oktay et al., 1997; Callejo et al., 2001; Radford et al., 2001). Antral follicles have developed as a result of exogenous hormone treatment in xenografts. Therefore, ovarian xenografting has the potential to allow for the study of follicular development in many species, while at the same time allowing for the study of ovarian reproductive abnormalities. Placing ovarian tissue into a recipient animal with a different estrous cycle length may allow for the study of follicular recruitment and development as well (Oktay et al., 1998).

In beef cattle, genetically superior cows are commonly treated with a hormone regimen that allows for excess follicular development to the antral stage. These extra antral follicles can be induced to ovulate, allowing for the release of multiple ova rather than a single ova. Spermatozoa are introduced into the reproductive tract of these cows to fertilize the ova and the developing embryos are collected 7 days later. These embryos can then be frozen or transferred into the uterus of a recipient cow. In either case, the maximal number of genetically superior offspring can be obtained from a single cow. However, these cattle typically stop ovulating prior to senescence. The reason for this phenomena is still unknown. It may be possible to investigate factors associated with

follicular development in the ovaries from cattle such as these if the ovarian cortical tissue were grafted into several mice. This would allow for different hormone treatments to be examined as well as allowing researchers the ability to study hormone interactions within this tissue and the ability of the ovarian tissue to express all factors, such as gonadotropin receptors, pertinent for folliculogenesis and ovulation. It may also be possible to collect multiple ova from tissue grown on the back of mice after xenografting tissue from the cow, thereby increasing the number of offspring a genetically superior cow may produce. When ovarian tissue is removed from the bovine, most of the follicles spontaneously differentiate from the primordial pool and enter the primary phase. This makes for the study of factors that initiate follicular development difficult but does allow for the study of inhibitors to follicular development.

By grafting ovarian tissue, the study of factors initiating follicular development and growth can be studied. To maximize the number of available ova in ovarian tissue, it would make sense to utilize ovarian tissue of fetal origin. Since the estrous cycle length in cattle is approximately 21 days and the mouse is approximately 4 days, studies need to be performed to determine if bovine ovarian tissue can successfully be grafted into mice and to determine if folliculogenesis can occur normally. The objective of this research was to determine if bovine ovarian cortical tissue of both fetal and adult origin will survive grafting onto the back of nude mice. Moreover, this research was undertaken to determine if grafted tissue spontaneously differentiates. The hypothesis was that follicles in grafted ovarian tissue would not spontaneously differentiate.

MATERIALS AND METHODS

Animals

The Washington State University Animal Care and Use Committee approved all animal procedures. Ovarian tissue was collected from cattle of different ages at a local abattoir. Nude mice (Taconic, Germantown, NY), aged 8-24 weeks were utilized as recipient animals and were housed at Washington State Universities experimental lab animal building under high efficiency particulate filtered microisolators. All mice had access to food and water ad libitum.

Collection of ovarian tissue

The ovaries were carefully dissected from slaughtered cattle of mixed breeding or from the fetus of these cattle. After removal of the ovaries, each ovary was placed directly into a sterile microcentrifuge tube (for fetal ovaries) containing sterile Hanks balanced salt solution (HBSS). Adult ovarian tissue was immediately dissected of the cortex in strips before being placed in a microcentrifuge tube containing sterile HBSS. Cortical strips were only taken from areas of adult ovarian tissue that was visually determined to not contain antral follicles. The ovarian tissue was maintained on ice prior to grafting

Surgery

At the time of surgery, mature female nude mice were anaesthetized with an intraperitoneal injection of a combination of ketamine (0.1 mg/kg body weight [bw]) and xylazine (0.05 mg/kg bw) in sterile physiological saline. Once the mice showed no pedal

reflex, the dorsal skin was swabbed with 70% ethanol followed by betadine. Ovariectomy was performed with a single 0.7 cm incision at the midline of the back with blunt dissection used to pass the muscle layer into the peritoneal cavity, the fat pad containing the ovary was located and externalized and the ovary was removed. This procedure was performed on the opposite ovary to complete the procedure. Following ovariectomy, 2 to 4 pieces of bovine ovarian cortical tissue were grafted under the skin of the mouse. Briefly, a small incision was made through the skin on the backs of the animal where the muscle was scored in individual areas, and four 2x2 mm to 3x3 mm pieces of donor bovine ovary were placed individually within the scored muscle areas of each recipient mouse. The incisions were closed with suture and the animals allowed to recover.

Experimental design

Bovine ovarian tissue from animals of different development ages was grafted under the skin of 32 nude mice. Each mouse received ovarian tissue from one donor animal. Vaginal smears were taken daily to determine estrous cycle activity. Mice were killed at 1 week, 2 weeks, 4 weeks, 12 weeks and 24 weeks following grafting. Grafts were recovered for determination of the extent of follicular development.

Graft recovery and histology

At the time of graft recovery, the animals were euthanized by CO_2 inhalation and cervical dislocation. The back was opened and the grafts were carefully removed. Recovered grafts were immediately placed in Bouin's solution for 24 hours at 4°C.

Samples were subsequently dehydrated with 3 washes of 70% alcohol, embedded in paraffin, sectioned at 7µm and stained with hematoxylin and eosin. All sections were analyzed using light microscopy (Seiler Microlux, Saint Louis, MS). The sections were examined for the presence of follicles. Follicular development was determined and classified as follows: Primordial follicles with one layer of flattened granulosa cells, primary follicles having one or two layers of cuboidal granulosa cells, or secondary follicles having three or more layers of granulosa cells but no antrum. Digital images were captured with a CoolSnap digital camera (Media Cybernetics, Silver Spring, MD).

Immunohistochemistry

Immunolocalization of follicle stimulating hormone receptor (FSHR) was performed in randomly selected samples from each time period of each donor age. Sections were deparaffinized in xylene, rehydrated in a series of ethanol washes, and heat-treated with a sodium citrate buffer to enhance antigen retrieval. Sections were preincubated with an endogenous peroxidase quenching solution (1 part 30% H_2O_2, 9 parts absolute Methanol) for 10 min at room temperature. The primary antibody, a 1:400 dilution of FSHR (0.5 µg/ml) (Santa Cruz Biotechnology, Santa Cruz, Ca) was added overnight in a humidified chamber at 4°C followed by a series of washes in PBS. The remaining steps are from a Histostain-sp kit (Zymed Laboratories Inc., San Francisco, Ca) with rabbit anti-goat biotinylated second antibody, HRP-streptavidin plus and a substrate chromogen mixture being utilized to cause color change. Once color change occurred, slides were rinsed and countestained with hematoxylin.

RESULTS

Vaginal Cytology

Vaginal smears from all intact mice demonstrated cyclic changes in vaginal cytology characteristic of estrous cycles. Cornified epithelial cells were seen at intervals of seven days in grafted mice. Vaginal smears in mice that had received s.c. ovarian grafts showed presence of cornified epithelial cells as early as six days after transplantation. After performing vaginal smears daily for several days, a thick mucous was discovered in the vagina of several mice and vaginal smears indicated sever leukocyte infiltration into the vagina, possibly indicating infection. These animals were dropped from the analysis and vaginal smears were discontinued.

Follicle survival and Development in Bovine Xenografts

Bovine xenografts were distinguishable from the surrounding host s.c. tissue and could be recovered in all but one grafted mouse. Follicular development did not get past the secondary stage in any of the xenografted tissue, in fact, most of the follicles were still in the primordial or primary stage (Figures 1 and 2). The exception to this was in fetal day 160 grafts that were removed after one week. This tissue contained a large amount of luteal tissue and very few follicles of any stage were visible. Much of the recovered tissue appeared to have developed fibrosis (Figure 1). Table 1 shows the number of follicles that were found in ovarian tissue of different age donors and different time periods after grafting. One mouse received a fetal day 80 bovine ovary which was

removed after four weeks. Interestingly, this mouse showed possible seminiferous cord development in the grafted tissue.

Presence of Follicle Stimulating Hormone Receptor

Follicle stimulating hormone receptor was located in bovine ovarian control tissue (Figure 3) and was localized to the granulosa cells of growing follicles that would be classified as large secondary follicles or larger. The negative control tissue lacking the primary antibody showed no staining. The appearance of FSHR in the majority of grafted tissue was not found as most of the tissue did not have follicular development beyond the primary follicle stage. The exception was tissue from 160 day fetal ovaries that were grafted for two weeks (Figure 4).

DISCUSSION

This study confirms the results of Derrar et al. (2000) who found that explants of bovine ovarian tissue did not spontaneously develop into primary follicles. These results confirm our hypothesis that xenografted bovine ovarian tissue would not have spontaneous activation of primordial follicles. Bovine ovarian tissue that was grafted into mice still had approximately 33% of the initial follicular pool remaining after 12 weeks and the majority of these follicles were of the primordial classification. In contrast, Braw-Tal (1997) showed that most primordial follicles in cortical slices of bovine ovaries spontaneously develop into primary follicles when cultured in vitro.

Bovine ovarian tissue was grafted into nude mice for 24 weeks with the expectation that antral development would occur as was seen when human ovarian tissue was grafts in SCID mice for more than 20 weeks (Gook et al., 2001). We hypothesize that fetal ovaries containing more ova would be better for studying development to the antral stage in grafted tissue. The limited number of follicles in the adult tissue, as a result of follicle turnover and age when compared to fetal tissue, may be the reason no follicular development was seen beyond the secondary stage. The adult bovine grafts were devoid of ova by the amount of time it too Gook et al. (2001) to see antral development in human ovarian grafts.

It is interesting that the total number of follicles of any stage in the fetal day 160 tissue appears to increase as the time grafted increases. Since different donor animals contributed the data to different time points, it may be that the ovarian tissue from one donor contained more follicles than from another donor. Another more likely possibility, would be that the area of ovarian tissue in which histology was performed differed in different donor animals, and that in one donor animal the area counted contained many follicles while the area counted in another donor did not. Performing histology on the total ovarian area may allow for a more accurate representation of follicle numbers. It is also important to note that the presence of luteal tissue at one wk accounted for the majority of the ovarian tissue. At two wk, the amount of luteal tissue was lessened, however, it was still present and thus inhibited our ability to visualize the presence of follicles in the ovarian tissue.

Fibrotic tissue was noted primarily in ovarian tissue from adult donor animals. This is similar to what was found with older humans (Kim et al., 2002). This was also

found in the Common Wombat ovarian tissue when it had taken too long between death of an animal and grafting of its ovaries (Wolvekamp et al., 2001). It is possible that the combination of animal age and time to grafting in this study was inhibitory to follicular survival. Ovarian tissue was collected at a local abboitoir and was grafted from seven to 14 h later suggesting this is too long for follicular survival.

In the single mouse that received ovarian tissue from a day 80 bovine fetus, what appeared to be seminiferous cords developed. This is similar to what was found in the tammar wallaby by Mattiske et al. (2002) when grafting ovaries from pouch young < 20 days of age. Taketo et al. (1993) indicated that fetal ovaries can be induced to form testicular cords after transplantation and that a loss of germ cells in the ovary can lead to the formation of seminiferous cords. It has also been suggested (Whitworth et al., 1996) that the loss of mitotic germ cells is enough to cause ovarian sex reversal. Unfortunately, we were unable to obtain more fetal ovarian tissue of suitable age to retest whether this result was an aberration. Further research should be performed to elucidate whether seminiferous cord development occurs in ovarian tissue of bovine fetal origin.

Table 1. Number of follicles counted in 3 sections at different time points of graft retrieval.

	Time 0	1 week	2 weeks	4 weeks	12 weeks	24 weeks
160 day	222 ± 12	15 ± 3	35 ± 9	62 ± 7	73 ±9	----
adult	20 ± 4	----	----	6 ± 3	4 ± 1	1 ± 1

Figure 1. Ovarian tissue from an adult bovine grafted into a nude mouse recipient for 12 weeks. Bar = 50 µm, magnification = 200x

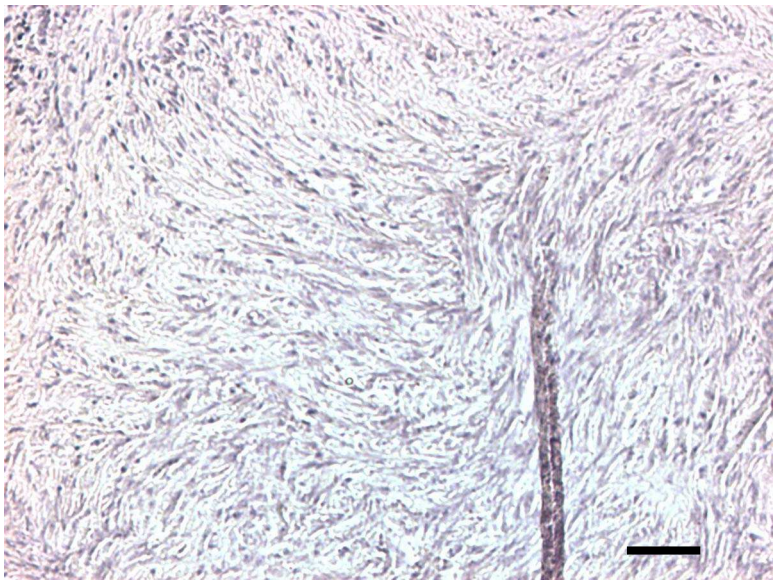


Figure 2. Day 160 fetal bovine ovarian tissue grafted into nude mice and removed 12 weeks later. The presence of many primordial and primary follicles is similar to that of fresh tissue. Stain = H and E, bar = 50 µm. Magnification = 200x.

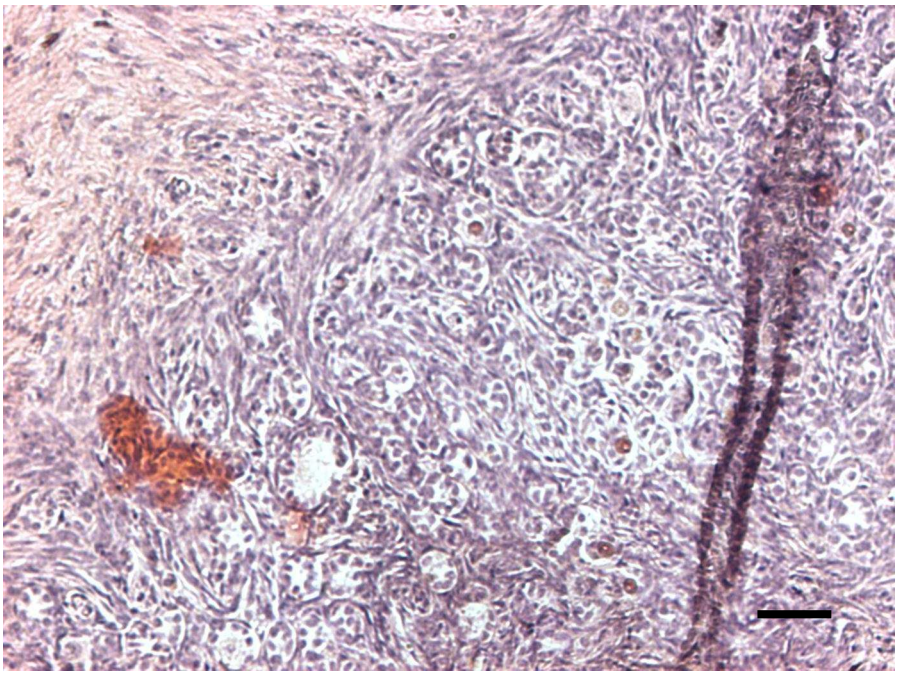


Figure 3. Immunohistochemical localization of FSHR in bovine control tissue. Red stain indicates FSHR containing cells, counterstained with hematoxylin. Magnification = 200x, Bar = 50 µm

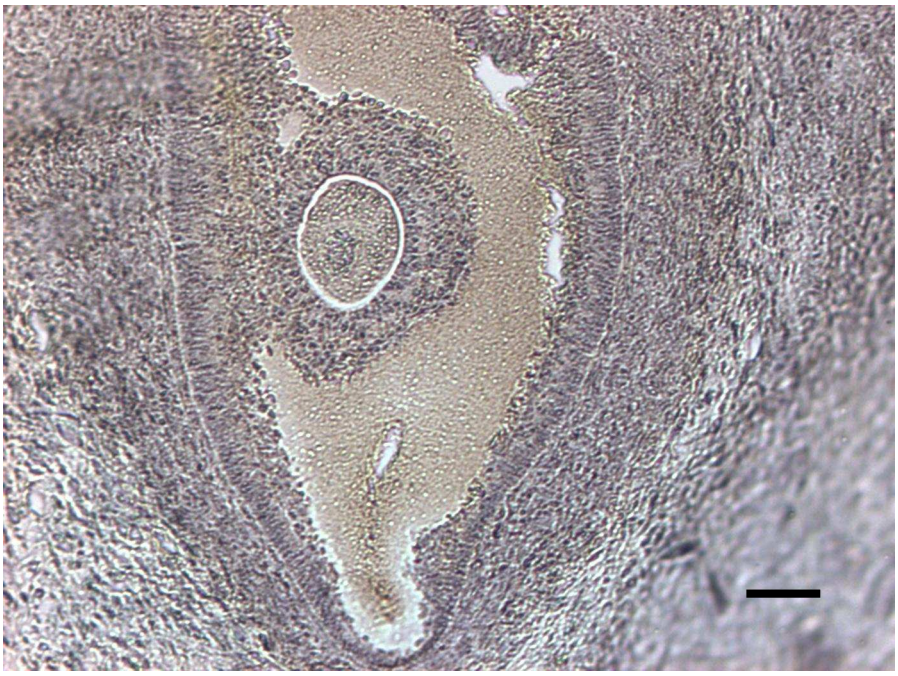
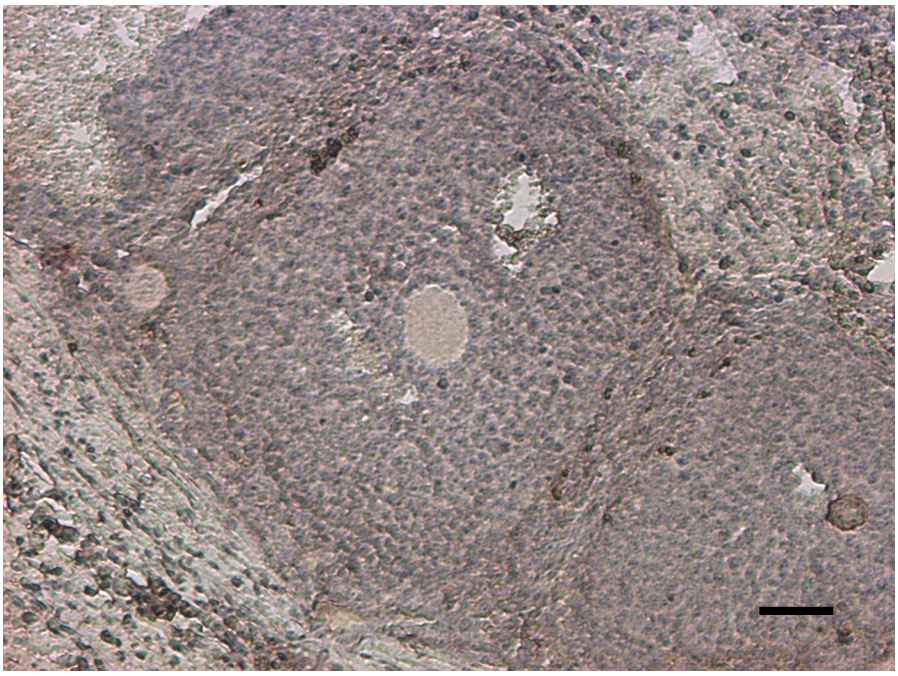


Figure 4. Immunohistochemical localization of FSHR in 160 day bovine fetal ovaries grafted into nude mice for 2 wk. The only large secondary follicles found during the study containing FSHR in the granulosa cells. Bar = 50 µm. Magnification = 200x



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