EFFICIENCY OF LHRH IMMUNIZATION AS A MALE STERILIZATION VACCINE

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To the Faculty of Washington State University:

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Abstract

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The objective of the studies presented herein is to examine the effectiveness of immunocastration using recombinant LHRH fusion proteins compared to surgical castration. In experiment 1, reproductive and hormonal effects of recombinant fusion proteins were investigated in Nelore bulls in Brazil. This experiment was divided into two studies. In study I, animals were separated into three treatment groups: intact; surgically castrated; and LHRH immunocastrated. LHRH immunocastrated bulls were immunized against a cocktail of two recombinant LHRH-7 fusion proteins. In study II, a fourth treatment group was implement in which half of the LHRH immunocastrated bulls received an additional immunization, and this study extended and additional year. LHRH antibodies were not detectable in the castrate or intact animals in either study. In both studies, scrotal circumference decreased as LHRH antibody binding increased in immunized bulls. By d 287 serum testosterone concentrations for all castrate groups in both studies was decreased when compared to intact controls. In experiment 2, carcass characteristics were examined for the bulls in study I. Average tenderness scores were inferior for intact bulls compared to immunized and castrated animals. Additionally, both castrated and immunized animals had greater marbling and percent carcass fat than the

intact bulls. The producer received premium steer price for LHRH immunized bulls over intact controls at the time of slaughter. These data support the concept that immunocastration of bulls at 2 yrs of age is successful in inducing a castrate like effect and has practical application as a tool for managing grass fattened bulls in Brazil. Experiment 3 was conducted to gain a deeper understanding of immunocastration on a molecular and cellular level. Mature Sprague Dawley male rats were divided into four treatment groups: intact, surgically castrated, LHRH immunocastrated, and rats which received both surgical and LHRH immunological castration. Interest in the cellular aspects of LHRH active immunization arises from the knowledge that although immunocastration and surgical castration are both effective methods used to suppress reproduction, the manner by which each is achieved is unique. Microarray analysis was utilized to examine gene expression changes in the two types of castration.

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Dedication

This dissertation is dedicated to my family who gives unselfishly of themselves and is a constant source of love and support in my life.

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INTRODUCTION

The cattle industry receives a large amount of scrutiny from animal welfare and animal rights groups as a result of surgical spaying and castrations without the use of anesthesia (Bonneau and Enright, 1995). Castration is used to produce animals with reduced sexual and/or aggressive behavior. These animals fatten more readily, are easier to manage and have the potential to reach market weight at an earlier age (Schanbacher and Ford, 1976; Schanbacher and Crouse, 1980). However, castration is laborious and can lead to mortality or mobidity (Bonneau and Enright, 1995). In male pigs surgical castration is economically undesirable, due to the growth retardation, reduced carcass quality and higher farming cost associated with less efficient feed conversion (Newell and Bowland, 1972; Walstra, 1974). Immunization with LHRH prevents reproductive function in male and female mammals and may be an alternative to surgical castration (Robertson et al., 1979).

Brazil produces twice as many cattle as the United States without using hormone implants to enhance growth. In this production system bulls rather than steers are fed on pasture for three years before being sent to slaughter. The approach used in Brazil is to utilize the animal's endogenous androgens, produced by the testis, to induce growth up to two years of age. At two years of age, the producer would like to castrate the bull to eliminate the aggressive behavior and acquire a higher quality carcass than that of intact bulls. However, a higher degree of trauma is associated with surgical castration at two years of age, and the potential for death loss increased by the presence of screwworms (*Cochliomyia hominivorax*) in Brazil. The use of a sterilization vaccine may be best realized this type of production system to avoid the problems associated with surgical castration at two years of age. Immunoneutralization of the

hypothalamic decapeptide LHRH had been shown to attenuate testicular function in bull calves (Adams et al., 1993, 1996) and may be an effective alternative to surgical castration. Vaccine inhibition of LHRH modulates testosterone concentrations and maintains growth characteristics while reducing the masculinity of the carcass (Adams et al., 1993; Cook et al 2000).

Few studies have examined the effect of immunocastration on gene expression in animals. Necessity to understand the regulation of a sterilization vaccine has led to examination of pituitary gene expression in immunocastrated and surgically castrated rats. The objectives of the research described in this dissertation is to identify the potential for use of an LHRH fusion protein vaccine in Brazil and to obtain a clearer view of how the LHRH fusion protein vaccine affects an animal on a cellular level.

LITERATURE REVIEW

Spermatogenesis

The reproductive capability of a male is dependent upon normal and functional spermatogenic development. Active immunization against LHRH has been shown to cause gonadal atrophy, which is related to disruption of spermatogenesis in cattle (Adams and Adams 1992; Cook et al., 2000), sheep (Jeffoate et al., 1982; Schanbacher et al., 1982; Kiyma et al., 2000), goats (Godfrey et al., 1996), and pigs (Molenaar et al., 1993; Meloen et al., 1994). Sperm production in the male involves the processes of cell division and differentiation by which spermatozoa are produced in the testis (Johnson et al., 2000). Normal spermatogenesis in mammals is dependent upon a functional hypothalamic-pituitary-testicular axis (Roser, 2001). Production of spermatozoa requires adequate production of luteinizing hormone-releasing hormone (LHRH) from the hypothalamus, followed by classic endocrine actions of the pituitary gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH), and feedback mechanisms of testosterone from the testis (Amann, 1983). Release of LHRH from the hypothalamus in the male occurs in frequent, intermittent burst that last for a few minutes and causes episodic secretion of LH and FSH from the pituitary gland (Irvine and Alexander, 1987). The episodic release of LH is not lasts between 10 and 20 minutes and can occur three to more than eight times per day (Hafs and McCarthy, 1979; Ketangole et al., 1971). Follicle stimulating hormone, also released under the influence of LHRH, concentrations are lower than those of LH but the duration of the pulses are longer due to the relatively constant secretion of inhibin by the adult testis (Purvis and Hansson, 1981; Walton et al., 1980). Luteinizing hormone leads to the subsequent production of testosterone by binding to membrane bound receptors in the Leydig cells of the testis, while FSH works through receptors on the Sertoli cell.

Leydig cells are large polyhedral cells that are found in clusters and are associated with the lymphatics and blood capillaries in the interstitial compartment of the testes (Setchell, 1978). Steroid production by a testis is correlated with the amount of smooth endoplasmic reticulum in the Leydig cells (Zirkin et al., 1980). Cholesterol is produced from acetate within the smooth endoplasmic reticulum of the Leydig cells (Amann and Schanbacher, 1983). Enzymatic cleavage of the cholesterol side chain by cytochrome P450 enzyme, cholesterol side chain cleavage (P450ssc) in the mitochondria results in the formation of pregnenolone (Payne et al., 1995). This is the rate-limiting step in the production of testosterone and formation of pregnenolone (Amann and Schanbacher, 1983). Pregnenolone is rapidly converted to testosterone by intermediates in the smooth endoplasmic reticulum (Ewing and Brown, 1977). Increase synthesis and pulsatile secretion of testosterone by the Leydig cells occurs in 30 minutes after the onset of an LH episode (Amann, 1983). Brown et al. (1994) demonstrated that intact rams that were challenged with LHRH had an increase in plasma LH concentrations within 10 minutes of the injection; followed by an increase in plasma testosterone within 20 minutes after the LHRH injection. Testosterone is the hormone in the male that is responsible for secondary sex characteristics such as masculine features, aggressive behavior as well as enhanced growth and production (Seideman et al., 1982; Cosgrove et al., 1996). Periodic production of testosterone by the Leydig cells increases intertesticular concentrations of testosterone 100 to 300 times the concentration in the peripheral blood (Amann and Ganjam, 1973). As a result of the three to eight daily episodes of testosterone, the seminiferous tubules are constantly bathed in fluid containing high concentration (Setchell, 1978), and this maintained high intratubular concentration of testosterone appears essential for spermatogenesis (Courot et al., 1979).

Estradiol is also involved in the hormonal regulation spermatogenesis. Production of estradiol in the testis is primarily from the Leydig cells, but testosterone is also converted to estradiol by the Sertoli cells (Purvis and Hansson, 1981; van der Molen et al, 1981). Testosterone and estradiol produced by the Leydig cells enter the peripheral circulation. These steroids in the blood reach the hypothalamus and pituitary gland, and exert a negative effect on the production of LHRH and the gonadotrophins (Amann, 1983). Consequently, high testosterone or estradiol concentrations in the serum result in a suppression of LHRH and LH.

Sertoli cells, the only somatic cells within the seminiferous tubules, play an important role in spermatogenesis and provide the microenvironment necessary for germ cell development (Amann and Schanbacher, 1983). The tubules are divided into two compartments by the tight junctions between adjacent Sertoli cells (Gilula et al., 1976; Russell and Peterson, 1985). Tight junctions are one component of the blood testis barrier, which effectively excludes serum macromolecules from entering the adluminal compartment of the Sertoli cells (Griswold, 1995). The number of Sertoli cells in the testis is established during puberty and remains constant in the adult male (Lino, 1971). In response to FSH and the availability of testosterone, Sertoli cells secrete androgen binding protein (ABP) and inhibin (Steinberger, 1981). In the seminiferous tubules of the epididymis the role of ABP is to diminish changes in testosterone concentrations or to aid in testosterone transport. Androgen binding protein also maintains high levels of testosterone within the seminiferous tubule. Inhibin suppresses FSH release from the anterior pituitary (Blanc et al., 1981; Ritzen et al., 1981).

Spermatogenesis takes place in the seminiferous tubule and is the sum total of the events that occur within the testis that result in spermatozoa production (Johnson, 1991). Spermatogenesis consists of three major divisions (spermatocytogenesis, meiosis, and

spermiogenesis). It is a process that involves production of spermatozoa while maintaining spermatogonial stores (Courot et al., 1970). A seminiferous tubule contains reserve A_0-spermatogonia that do not enter into the proliferating pool of germ cells (Hochereau-de Reviers, 1981). Spermatocytogenesis consists of committed A_1-spermatogonia proliferating by mitosis and giving rise to differentiated A_2-spermatogonia and these cohorts increasing differentiated in unison to ultimately form primary spermatocytes (Amann, 1983; Amann and Schanbacher, 1983). The basal compartment of the Sertoli cell contains all spermatogonia plus preleptotene and leptotene spermatocytes (Amann, 1983). The developing primary spermatocytes pass through the tight junctions between Sertoli cells to enter the adluminal compartment (Dym and Fawcett, 1970; Setchell, 1978). Within the adluminal compartment, the spermatocytes continue through meiosis, divide to form secondary spermatocytes, and ultimately become spermatids after complete meiosis (Amann, 1983). Meiosis involves the reduction of chromosome to the haploid state and it ensures genetic diversity. Spermiogenesis is the morphologic transformation of spherical spermatids into spermatozoa (Johnson et al., 2000). Isolation of the meioticly dividing germ cells and spermatids during their transformation to spermatozoa in the adluminal compartment is essential for their development (Setchell, 1978).

The cycle of the seminiferous epithelium is the progression through cellular stages at one location along the seminiferous tubule. The more immature cell types are located near the basement membrane while the more advanced cell types reside in the adluminal compartment. The cycle of the seminiferous tubule is species specific and takes 13.5 days in the bull (Johnson et al., 2000). During spermatogenesis, cells at a given area of the seminiferous epithelium proceeds through 4.5 cycles of the seminiferous epithelium (Amann, 1970). Therefore, in the bull the complete process from A-spermatogonia to a fully differentiated spermatozoa takes 61

days (Johnson et al., 2000). The spermatogenic wave refers to the spatial orientation along the length of the seminiferous tubule that changes over time. During the wave, each stage of the seminiferous tubule becomes a successively more advanced stage. The wave allows for a relatively constant supply of spermatozoa in the epididymis, creating a constant pool of sperm for ejaculation (Senger, 1997).

Hypothalamic Pituitary Gonadal Axis

Mammalian reproduction is controlled by a cascade of endocrine events that begins with the secretion of LHRH from neuronal cell bodies in the hypothalamus. The hypothalamus is located at the base of the brain and is composed of clusters of neurosecretory cells called hypothalamic nuclei which secrete their products either into the portal blood system that connects the hypothalamus to the anterior pituitary or directly into the general circulation after storage in the posterior pituitary (McCann and Ojeda, 1996). Hypothalamic hormones are classified as releasing or inhibiting hormones because of the nature of their action (McCann and Ojeda, 1996). Luteinizing hormone releasing-hormone (LHRH), also known as gonadotropin hormone releasing-hormone because of its ability to stimulate both LH and FSH release is a 10 amino acid peptide (Jennes and Conn, 1994). The primary structure of LHRH (pyroGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) was determined in 1971 using extracts from porcine hypothalami (Matsuo et al., 1971). LHRH is produced as a portion of a precursor molecule, which includes a 23-amino-acid signal sequence, the LHRH decapeptide, and a 56-amino-acid LHRH-associate peptide (Seeburg and Adamek, 1984). This hormone is a highly conserved decapeptide that is synthesized mainly in the anterior hypothalamus and medial preoptic area of the mammalian brain (Silverman et al., 1979; King et al., 1984). LHRH is synthesized from neural cell bodies located in the preoptic area, it is packaged into secretory granules and

transported intraaxonally to neurovascular terminals in the median eminence where it is released (Jennes and Conn, 1994; King and Letourneau, 1994). Axons from the cell bodies of the hypothalamic neurons extend into the pituitary stalk region where the nerve endings terminate and release LHRH into the fenestrated capillaries and is carried via the hypothalamo-hypophyseal portal system to the anterior pituitary to regulate gonadotrophin secretion (Smith and Jennes, 2001). The hypothalamo-hypophyseal portal system allows minute quantities of LHRH to be released onto the adenohypophysis before dilution by systemic circulation. The releasing hormone is then transferred to a second capillary bed in the anterior pituitary triggering the release of LH and FSH from the gonadotroph cells. LHRH neurons are the last output pathway of the neural system that incorporates numerous internal and environmental cues to regulate the secretion of LH and FSH from the anterior pituitary (Smith and Jennes, 2001).

The pituitary gland consists of the neurohypophysis that is of neural origin and the adenohypophysis of ectodermal origin. The adenohypophysis lacks functional innervation from the hypothalamus or neural lobe, instead communication is provided by means of the hypophyseal portal system of veins (McCann and Ojeda, 1996). Knowledge of hypothalamic control of the pituitary has come primarily from studies involving hypothalamic lesions or stimulation in animals. All hormones produced by the adenohypophysis are protein or polypeptide in nature (McCann and Ojeda, 1996). LH and FSH are glycoproteins secreted by the same basophilic cells in the pituitary gland. The pituitary gonadotropes make up 8-15% of all the cells in the anterior pituitary (Norwitz et al., 1999). These gonadotropins are comprised of two subunits including a common α and unique β subunit. Each subunit is encoded by a separate gene and is contained on a separate chromosome (Gharib et al., 1990). Gonadotropes consist of 60% mulithormonal cell, which produce both LH and FSH, and 18% LH and 22% FSH

containing cells (Shacham et al., 2001). Immunization against hypothalamic or pituitary hormones has been examined in numerous studies as a means to effect reproduction.

Immunomodulation Through Active Immunization

The immune system has the ability to remember past infections and respond aggressively upon reintroduction to a previous pathogen. This immunological memory is the basis upon which vaccinations are possible (Kim and Flavell, 2004). The primary function of the immune system is to discriminate between 'self' and non-self and respond specifically to molecules identified as foreign (Simpson, 1984). 'Self' describes substances belonging to the genetically normal structure and function of an organism (Hunter, 1989). Those substances that are identified by the immune system as foreign or non-self, and have the ability to induce specific immune reactions are antigens. In response to antigens an organism may produce antibodies that react specifically with an antigen (Hunter, 1989).

The rejection of foreign or non-self antigen is mediated by genetic loci that form a region known as major histocompabilility complex (MHC) (Roitt et al., 1998). All mammals have an MHC locus that codes for glycoproteins expressed on the cell surfaces (Simpson, 1984). MHC proteins are markers for self and are unique to each individual (Hunter, 1989). During their development, immature T cells are selected for their ability to interact with self MHC, in a process called positive selection. (von Boehmer, 1994). After antigen-specific T cell receptors are aquired, all T cells reacting with self-antigens are eliminated by apoptosis, this plays a major role in self-tolerance (Nossal, 1994).

Immunity is dependent on both nonspecific (innate immunity) and specific immunity (adaptive immunity). Innate immunity is comprised of defensive barriers including anatomic,

physiologic, phagocytic and inflammatory and is the first line of defense against a pathogen. Adaptive immunity on the other hand displays a high degree of specificity as well as "memory". Immune responses can be divided into the humoral and the cell-mediated responses (Simpson, 1984). Humoral immunity refers to antibody responses or the manufacture and secretion of immunoglobulins by B lymphocytes that are carried through the body via the blood circulation and tissue fluids (Simpson, 1984). Cell-mediated immunity involves a group of responses carried out or initiated by sub-populations of T cells (Hunter, 1989). These responses are interconnected and the presence of each type of response to determinants on the same antigen modifies the other (Simpson, 1984).

T lymphocytes are responsible for coordinating a highly specific and long lasting immune response to pathogens (Fabbri et al., 2003). Long-term immune protection is mediated by memory T and B cells as well as antibody producing plasma cells (effector B cells) (Kim and Flavell, 2004). In response to an invading pathogen, T cells specific for foreign antigen work to clear the infection by expanding and differentiating into effector cells (Mackay and von Andrian, 2001; Kim and Flavell, 2004). In addition to differentiating into effector B cells that kill pathogens, T cells stimulate B cells to make antibody against the foreign antigen; others become memory cells that may differentiate into effector cells upon future encounters with the antigen (Mackay and von Andrian, 2001). Specificity of recognition, memory of previous antigen encounter, and lack of self-reactivity of the T cell are features acquired during maturation in the thymus (von Boehmer, 1997). T cells are derived from lymphoid precursors produced in the bone marrow and differentiated in the thymus, where they develop the ability to distinguish self from non-self (Fabbri et al., 2003). The T cell receptor, acquired through gene rearrangement during development in the thymus, is responsible for single antigen specificity for the life of the

T cell (Fabbri et al., 2003). The T cell receptor works by recognizing antigens presented as processed peptides that are loaded on the major histocompatability complex (MHC), which is expressed on the antigen presenting cell plasma membrane (Viret and Janeway, 1999). Individual subsets of T lymphocytes orchestrate the overall immune response through paracrine cell signaling (helper $CD4^+$ T cells), while cytotoxic $CD8^+$ T cells directly eliminate invaders (Fabbri et al., 2003).

T cell-mediated immune response occurs in stages beginning with the naïve T cell. Naïve T cells are those, which have never encountered antigens. Adaptive immunity relies on each naïve T cell bearing a unique antigen receptor capable of recognizing antigens presented to them by other cells (Fabbri et al., 2003). Innate immunity involves cells, such as dendritic cells, that directly recognize a limited set of "pathogen-associated molecular patterns" and are responsible for the preliminary trapping of the antigen in the periphery and its presentation to T cells in the lymph nodes (Janeway and Medzhitov, 2002). T cells with the CD4 membrane marker, function in the generation, maintenance and regulation of both the humoral and cellular arm of the immune response (Flavell, 1999), and are defined as "helper" (Th) cells. T-helper cells are divided into two major (Th1 and Th2) subsets based on their pattern of cytokine production and associated function (Roitt et al., 1998; Sallusto et al., 1998). The Th1 subset secretes interleukin 2 (IL-2) and interferon-γ (IFNγ) and are involved in cell-mediated inflammatory reactions while Th2 cells secrete IL-4, IL-5, IL-6 and IL-10 and are more effective at stimulating B cells (Cox and Coulter, 1997; Singh and O'Hagan, 1999). B cells, like T cells must be tightly regulated to prevent non-specific activation of cells during an infection (Carroll, 2004). Antigen specificity is insured by the requirement for B cell receptor and CD40, to promote activation and expansion against specific pathogens (Carroll, 2004). Adaptive

immunity is characterized by the formation of memory B cells, which are antigen selected and demonstrate high affinity (Carroll, 2004). Th1 and Th2 responses are mutually inhibitory (Mosmonn and Moore, 1991) and effective immunity is dependent of the appropriate induction of a Th1 or Th2 response (Singh and O'Hagan, 1999). Conversely, differentiated CD8 T cells are defined as cytotoxic as they kill pathogen-infected cells by activating an apoptotic program (Fabbri et al., 2003).

Adjuvants are also capable of enhancing Th responses. An adjuvant is any substance that acts to accelerate, prolong or enhance the quality of the immune response to vaccines (Vogel, 2000). The effect of the adjuvant is due in large part to its ability to concentrate the antigen in a site where lymphocytes are exposed to it ('depot' effect) and the induction of cytokines that regulate lymphocyte function (Marciani, 2003). The use of adjuvants to improve vaccine efficiency was first described by Ramon (1925) who reported that antitoxin titers were higher in horses that developed abscesses at the site of injection of diphtheria toxin than in animals without abscesses. A decade later Freund (1937) developed what is still one of the most effective adjuvants known; termed Freund's adjuvant. Freund's complete adjuvant (FCA) is a water-in-mineral-oil adjuvant, which combines the immunostimulatory properties of *Mycobacterium tuberculosis* along with a short-term depot effect (Cox and Coulter, 1997; Vogel, 2000). This adjuvant generates very strong Th1 and Th2 responses (Cox and Coulter, 1997).

'Self' antigens without any adjuvant will only rarely induce useful immune responses (Meloen, 1995). Often, such responses are not optimal and may require larger doses and multiple injections (Meloen, 1995). For this reason 'self' antigens are usually used in combination or emulsified with an adjuvant to enhance the immune response (Meloen, 1995). Adjuvants provide a reservoir for the antigen allowing for slow release into circulation, thus

continually stimulating the immune system. Additionally, these immunostimulants produce a local inflammation that activates, in a non-specific manner, the immune system for numerous responses including induction of cytokines and recruitment of cells (Dalsgaard et al., 1990; Stevens, 1993; Singh and O'Hagan, 1999).

Active Immunization Against LHRH

Vaccination against LHRH has been studied intensely over the years because of its potential to shut off the reproductive axis completely (Meloen, 1995). A problem with using 'self' antigens, like LHRH, is that they are poorly immunogenic (Reeves et al., 1989). Various methods have been used to overcome the lack of immunogenicity of a 'self' antigen. One such method involves coupling of the antigen to a carrier molecule such as bovine serum albumin (BSA), tetanus toxoid (TT) or keyhole limpet hemocyanin (KLH) (Meloen, 1995). This strategy for preparing antigens against small self-peptides is accomplished by synthesizing the peptide chemically and then conjugating it to a larger carrier protein using a coupling agent such as carbodiimide (Fraser et al., 1974). These carrier molecules function to effectively increase the decapeptide like LHRH thus allowing the hormone to be recognized as sufficiently foreign to elicit an immune response (Meleon, 1995). Changing the native structure of a molecule also provides an effective way to induce the immune system to respond to the altered antigenic site of a 'self' molecule by producing antibodies that cross react with the native molecule (Talwar et al., 1976). Applying a tandem repeat of an amino acid sequence to a small molecule like LHRH further increases the immunogenicity of the molecule (Oonk et al., 1993; Meloen et al., 1994).

Conjugated LHRH Vaccine

Adams and Adams (1986) noted that active immunization against LHRH serves as a method to introduce an immunological barrier between the hypothalamus and the anterior pituitary gland that specifically neutralizes LHRH in the hypothalamo-hypophyseal portal vasculature. Arimura et al. (1973) immunized rabbits with a synthetic LHRH conjugated to bovine serum albumin (BSA) and was the first to note that LHRH immunization suppressed gonadal function. Production of anti-LHRH in rabbits for the development of a LHRH radioimmunoassay was associated with gonadal atrophy in those rabbits. Since this report active immunization against LHRH was shown to suppress secretion of the gonadotropins, induce atrophy of the gonads, inhibit gametogenesis and block reproductive behavior in several mammalian species (Schanbacher et al., 1983; Adams and Adams, 1986; Falvo et al., 1986).

Rodents

A variety of approaches were investigated to determine the most effective method for immunoneutralization of LHRH. The first generation of vaccines was conjugation vaccines. These vaccines consisted of coupling LHRH to large, non-self proteins such as albumin, keyhole limpet hemocyanin, and diphtheria or tetanus toxoid (Fraser et al., 1974; Johnson and Tang, 1988; Hoskinson et al., 1990) to obtain an antibody response. Ladd et al. (1990) used LHRH linked to tetanus toxoid at the ten position (C-terminal) to successfully immunize male rats. The magnitude of response to the vaccine did not depend on dose of the antigen once a threshold dose of 50 :g had been reached. However, rats and rabbits immunized with LHRH conjugated to tetanus toxoid at the one position (N-terminal) had antibody concentrations sufficient to suppress LH and FSH and subsequently testosterone concentrations quicker than LHRH

conjugated at the six or ten position (Ladd et al., 1990). LHRH conjugated to tetanus toxoid resulted in decreased testicular size and testosterone levels in immunized rats (Shastri et al., 1981). Histological changes in the testes of Wistar rats occurred with immunization of LHRH conjugated to diphtheria toxoid and indicated arrest of spermatogenesis at the spermatocyte level and atrophy of in the Leydig cells (Giri et al., 1990). Exogenous androgen supplementation administered simultaneously with LHRH immunization caused infertility in rats while normal libido was maintained (Ladd et al., 1989). Nett and Adams (1977) demonstrated in rats that LHRH antibody titers were associated with reduced LH, FSH and testosterone concentrations and atrophy of testes, epididymides and seminal vesicles. Furthermore, no sperm was found in the testes and epididymides and seminal vesicles were devoid of luminal fluid. Prostate gland size also decreased in rats immunized against LHRH (Jayashankar et al., 1989).

Reversibility of suppressed pituitary-gonadal functions in male rats was achieved simply by allowing anti-LHRH antibody titers to decline (Ladd et al., 1989). In response to the declining antibody titers, the weights of the testes and epididymides were restored and fertility and spermatogenesis also returned to normal.

Bulls and Rams

Castration improves the behavioral traits of male cattle by decreasing the frequency and intensity of sexual and aggressive displays (Baker and Gonyou, 1986). Beef bulls (Adams and Adams, 1992) and ram lambs (Schanbacher, 1982) actively immunization against LHRH results in decreased LH secretion and, ultimately, suppression of both testicular development and testosterone concentrations (Schanbacher, 1982; Adams et al., 1993, 1996). Sexual behaviors, such as mounting frequency and ejaculations, were reduced in ram lambs immunized against

LHRH using FCA (Kiyma et al., 2000). Reports on immunization of bull calves against LHRH showed that appropriate titers of anti-LHRH could results in a reduction of testis size and serum testosterone concentrations and decreases in the magnitude of aggressive behavior (Jeffcoate et al., 1982; Robertson et al., 1981; Huxsoll et al., 1998). Moreover, reduction of LHRH can reduce serum testosterone concentrations allowing for good growth performance while reducing carcass masculinity (Adams et al., 1993).

Carcass quality is a major concern for producers and a primary reason for castration of males. In addition to the difficulty posed by the aggressive behavior of intact males, carcass quality suffers. High activity levels in post-pubertal bulls, boars, and heifers in estrus leads to a high tendency of these animals to yield bruised or dark-cutting meat at slaughter (Kenny and Tarrant, 1988; Kempster and Lowe, 1993). Suppression of testicular development by active immunization against LHRH has a marked effect on carcass quality. Adams and Adams (1992) showed that active immunization of steers and bulls with LHRH-keyhole limpet hemocyanin conjugate induced elevated antibody titers and reduced LH secretion for more than 28 weeks with either a primary immunization alone or primary and secondary immunizations. Feedlot performance and carcass quality were not affected. Immunoneutralization of LHRH in ram lambs reduced testis weight and the concentration of testosterone at slaughter (Kiyma et al., 2000). Feed efficiency of intact rams was greater than that of castrated or immunized lambs, but feed efficiency did not differ between immunized and castrated lambs. Immunization against LHRH did not affect feed intake of crossbred bulls, but bulls with the highest anti-LHRH responses tended to have carcass composition similar to that of steers (Lobley et al., 1992). However, intact and immunized ram lambs had more desirable yield grades, less backfat, and less marbling than castrated rams (Kiyma et al., 2000). The difference in partitioning of

nutrients for growth and deposition of fat may be due to the residual testicular activity in immunized rams (Kiyma et al., 2000). The reduction in carcass quality and performance associated with castration is partially reversed by administration of anabolic steroids to steers (Adams and Adams, 1992). However, steroid supplementation does not affect the feedlot performance or carcass characteristics, except for yield grade, in bulls immunized against LHRH (Adams and Adams, 1992; Huxsoll et al., 1998). The castration effect of male cattle immunized against LHRH was reversed within six months after cessation of immunization (Robertson et al., 1982).

Age at immunization appears to play a role in the effectiveness of the vaccine. Prepubertal transition and testicular development begins after 4 months of age in cattle and is associated with a marked increase in LHRH secretion (Rodriguez and Wise, 1989, 1991). During the peripubertal period, from 8 to 16 months of age, aggressive behavior of bulls is markedly increased (Baker and Gonyou, 1986; Price and Wallach, 1991) and is likely the result of an increase in testosterone production during this stage of development (McCarthy et al., 1979; Staigmiller et al., 1985). Dykeman et al. (1982) reported an eight-fold increase in the aggressive behavior of steers receiving exogenous testosterone compared to steers receiving vehicle alone. Immunization of adult bulls against LHRH produces only temporary suppression of gonadal function and sexual behavior that is reversed after antibody titers decline (Robertson et al., 1982; Keeling and Crighton, 1984). In contrast, Brown et al. (1994) showed that immunization of rams very early in life produced long-lasting effects on their reproductive capacity, with some rams having juvenile-like organs and complete lack of libido two years following immunization. Adams et al. (1996) demonstrated in bull calves that a primary immunization alone at 7 months of age resulted in greater suppression of testicular function and

size at 18 months of age as compared to bulls immunized at 1.5 or 4 months of age, although most carcass characteristics were unaffected. A similar study by Jago et al. (1997) reported that Friesian bulls immunized at 2.5, 4, or 7.5 months of age resulted in a delay in development of sexual and social behaviors, and that there was no practical reason for immunization earlier than 7.5 months. Beef bulls receiving a primary immunization at 1, 4 or 6 months of age and a single booster at 12 months of age demonstrated feedlot gain similar to control bulls, whereas aggressive behavior was reduced and marbling score and quality grade was improved regardless of age at first immunization (Huxsoll et al., 1998).

Boars

Although intact boars, like bulls, are more efficient and produce a leaner carcass than castrated animals they pose the unique problem of boar taint (Newell and Bowland, 1972). During sexual development and when mature, boars accumulate androstenone and skatole in their fatty tissue. These two substances are considered the main contributors to boar taint (Bonneau, 1982). Following castration in the boar, levels of androstenone (Bonneau, 1982) and skatole (Friis, 1993) in the fat decline. Immunization against LHRH could provide an alternative method to castration that is still effective in reducing boar taint in boars. Falvo et al. (1986) demonstrated, for the first time, that active immunization against LHRH is feasible and practical as an alternative to surgical castration in boars. This study also reported that immunization had no effect on average daily gain, hot carcass weights or loin eye area. Castration-like carcass effects and reduction of boar taint are observed in boars immunized against LHRH (Falvo et al., 1986; Dunshea et al., 2001). Bonneau et al. (1994) reported that immunization of young intact male pigs reduced genital tract weight and androstenone

concentrations in fat with only a limited effect on the performance of the animals. Immunization of sexually mature boars against LHRH conjugated to bovine serum albumin five times over a 24-wk period induced production of LHRH antibodies and reduced serum LH concentrations, testis volume, paired testis weight, paired epididymal weight, sperm per testis and seminiferous tubule diameter. These results suggested that both steroidogenic and spermatogenic functions were impaired in testes of mature boars actively immunized against LHRH (Grizzle et al., 1987).

A majority of studies investigating immunization against LHRH in male pigs have used a monomer of the native LHRH sequence conjugated to a carrier protein. This method generally does not induce an adequate response in all immunized animals. Alternatively, Meloen et al. (1994) used a tandem repeat of the native amino acid sequence of LHRH coupled to keyhole limpet hemocyanin (KHL) and vaccinated pigs twice with an 8-wk interval. At slaugher animals immunized with LHRH tandem repeats had decreased testicular size and undetectable testosterone concentrations and fat androstenone. The monomer sequence of LHRH conjugated to KHL was not as effective in decreasing testicular size, testosterone concentration or androstenone.

Recombinant LHRH Vaccine

Since conjugated vaccines lack consistent uniform molecular structure within and between batches, an alternative strategy that circumvents this problem has been developed in the use of recombinant fusion protein vaccines. Fusion proteins are constructed by first selecting a gene that codes for a carrier protein that can generate a strong antibody response (Margalit et al., 1987). Then an oligonucleotide, like LHRH, is synthesized and that piece of DNA is ligated to the gene of the carrier protein by placing it at the 5’ or 3’ end or at internal positions in the same

gene as directed by specific restriction sites in the DNA sequence of the carrier protein (Manns et al., 1997). Similar to other techniques used to increase immune response, approaches using recombinant proteins appear to be the most effective for generating immunogenicity when LHRH is present as repeating units (van der Zee et al., 1995; Manns et al., 1997; Zhang et al., 1999). Such a DNA sequence coding for a chimeric protein could then be expressed in a highly regulated bacterial expression system allowing for large amounts of recombinant proteins to be produced (Manns et al., 1997).

The use of conventional chemical conjugation of LHRH to carriers has also proved to be very expensive and results in an unpredictable ratio of antigen to carrier protein (Goubau et al., 1989). Thus, development of a recombinant vaccine has been studied for effectiveness as a new method to immunoneutralize livestock. Rozell (1993) used a recombinant ovalbumin vaccine containing three LHRH inserts in rabbits, but was only successful in inducing anti-ovalbumin, and not anti-LHRH antibodies. A possible explanation for the lack of the desired antibody response may be in part due the relatively small percentage that the ten amino acid LHRH peptide is of the overall recombinant protein. In 1995, van der Zee and colleages constructed a carrier system that was based on P-fimbriae of E. coli, which are filamentous cell-surface structures composed of repeating major sequences of LHRH. LHRH was inserted in the hypervariable region of P-fimbriae of E. coli by recombinant DNA technology. Without affecting fimbriae formation, the foreign antigen in the recombinant fimbiae was presented as repeating units (500-1000 copies per fimbriae), which could be advantageous for immunogenicity (Broeckhuysen et al., 1987). This vaccine proved effective in inducing high anti-LHRH titers and suppressing testicular growth in young bull calves (van der Zee et al.,

1995). Female mice vaccinated with purified recombinant LHRH fimbriae demonstrated high anti-LHRH titers and suppression of the estrous cycle (van der Zee et al., 1995).

Zhang et al. (1999) used 50 μg of the recombinant ovalbumin protein with seven LHRH (ovalbumin LHRH-7) inserts emulsified in FCA and one booster using FIA to decrease uterine-ovarian weight in immunized mice. Similarly, Sosa et al. (2000) reported immunization of heifers using the ovalbumin LHRH-7 fusion protein vaccine was effective in suppressing estrus from 60-238 d. Vaccinating bulls with a recombinant fusion protein anti-LHRH, with 8 LHRH copies fused to the carboxyl and amino ends of a leukotoxin carrier protein, suppressed testicular function while growth and carcass characteristics were similar to that expected with steers (Cook et al., 2000).

Development of an additional LHRH-7 recombinant protein was produced using the bacterial protein, thioredoxin (Quesnell et al., 2000). Administration of thioredoxin LHRH-7 alone or in combination with the ovalbumin LHRH-7 fusion protein vaccine was effective in decreasing the size and weight of vesicular glands, and prostate in male mice. Endocrine and carcass characteristics were evaluated in bulls that were administered a combination or 'cocktail' of both ovalbumin LHRH-7 and thioredoxin LHRH-7. Bulls vaccinated against LHRH fusion protein cocktail had suppressed testosterone and LH concentrations, while demonstrating growth and carcass characteristic similar to castrated steers (Aïssat et al., 2002)

Summary of Literature Review

The results of the recombinant protein anti-LHRH vaccine in combination with Freund's complete adjuvant are encouraging as a sterilization method for livestock. Use of such a vaccine may first be realized in foreign countries that produce larger numbers of cattle or swine. The objective of the studies presented in this dissertation is to evaluate the effectiveness of LHRH fusion proteins as an alternative to surgical castration methods in Brazil. Additionally, since there is a lack of information regarding the cellular effects of the vaccine we also investigate the effects of LHRH fusion protein vaccination on gene regulation using microarray techniques.

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CHAPTER TWO

Reproductive Characteristics of Grass Fed LHRH immunocastrated *Bos indicus* bulls

Reproductive characteristics of grass fed LHRH immunocastrated *Bos indicus* bulls

ABSTRACT: Two field trials were conducted in Brazil to evaluate LHRH immunocastration of *Bos indicus* bulls (d 0 = 2 yrs of age). In study I, 72 bulls were randomly assigned to one of three treatment groups: immunized, castrated and intact. Immunized animals (n = 25) received a primary and two booster injections of LHRH fusion proteins on d 0, 141, and 287. Twenty-three bulls were surgically castrated on d 141, and 24 served as intact controls. All animals were slaughtered on d 385 approximately 3 yr of age. In study II, 216 bulls were randomly assigned to the same three treatments as in study I. However, due to a drought in the area the producer decided to keep his bulls on pasture an additional year. Consequently, a fourth treatment was added in which one half of the LHRH immunized bulls received an additional booster on d 639 (4^{th} immunization), with all animals being slaughtered on d 741 (4 yrs of age). LHRH antibodies were not detectable in the castrate or intact animals in either study. In both studies, scrotal circumference decreased as LHRH antibody binding increased in immunized bulls. By d 287 serum testosterone concentrations for immunized bulls in both studies was decreased when compared to intact controls ($P < 0.01$). In study II, testes and epididymidal weights (g) at slaughter were higher ($P < 0.01$) for intact (500 ± 17 and 60 ± 2, respectively) than immunized bulls (173 ± 22 and 26 ± 2, respectively) and 4^{th} immunization bulls (78 ± 23 and 20 ± 2, respectively), the same significant effect was seen in study I. At the end of each study, BW was higher ($P < 0.01$) in the intact bulls compared to the castrated and immunocastrated animals. In the two studies the efficacy of this LHRH fusion protein vaccine to induce castration effects at slaughter was considered 92 and 93%, respectively. These data support the concept that

immunocastration of bulls at 2 yrs of age was successful and has practical application as a tool for managing grass fattened bulls in Brazil.

Keywords: LHRH, Immunocastration, Bos indicus, Bulls

Introduction

Active immunization against the hypothalamic hormone LHRH has been long recognized as a key hormonal target for sterilization in livestock. Arimura et al. (1973) conducted the first immunoneutralization study of LHRH and noted the subsequent reduction in the pituitary gonadotropins, LH and FSH. The decline or absence of LH and FSH leads to atrophy of the gonads and impairment of reproductive function (Adams et al., 1996; Miller et al., 2000). In the male rat, suppression of the gonadotropins as a result of LHRH immunocastration has been shown to induce complete azoospermia indirectly by suppressing the secretion of testosterone (Awoniyi et al., 1992). Since typical male characteristics of the bull such as aggression and superior growth are attributed primarily to the endogenous production of testosterone (Seideman et al., 1982; Cosgrove et al., 1996), ablation of testosterone by immunocastration results in an effect similar to surgical castration.

Surgical castration remains an important tool in beef cattle management to reduce aggressive behavior and improve management ease. However, numerous disadvantages are associated with castration, including the potential for morbidity and mortality (Bonneau and Enright, 1995), and the associated reduction in growth rate (Huxsoll et al., 1998). In a country such as Brazil where the probability for death loss is exacerbated by the presence of screwworms, bulls are left intact and fattened on grass for 3 yr. Since hormone implants are not

allowed in Brazil the androgenic effect of the testes for improved growth is especially important. Two years of age would be the desired age to castrate the bulls to suppress aggressive behavior since they are grazed in large groups of up to 500 bulls. However, surgical castration at this age is extremely traumatic while an immunocastrastion could accomplish the goals without the estimated loss due to surgical castration. The present two studies were designed to examine the effectiveness of immunocastration with LHRH fusion proteins on 2-yr old pasture fed bulls raised in the state of Mato Grosso, Brazil.

Materials and Methods

Preparation of Antigen

Fusion proteins ovalbumin-LHRH-7 and thioredoxin-LHRH-7 were prepared as previously described (Zhang et al., 1999; Quesnell et al., 2000). Ovalbumin-LHRH-7 contains seven LHRH sequences inserted at four different positions in the ovalbumin gene fragment, while thioredoxin-LHRH-7 contains seven LHRH sequences inserted at three distinct positions of the thioredoxin gene. The *E. coli* strain BL21(DE3) was used to express the proteins. Each gene construct expressed a 6-histidine sequence (His-tag, Novagen, 1994) at the carboxyl terminus to facilitate purification of the LHRH fusions proteins via nickel affinity chromatography. Purified proteins were combined at an equal molar basis to yield a total of 1.5 mg of protein per injection. The LHRH fusion proteins were emulsified in a water-oil adjuvant containing *Mycobacterium butyricum* for the primary immunization and the bacterial proteins were excluded from the adjuvant for booster injections. Shipment of the anti-LHRH vaccine was

approved by the Brazilian Ministry of Agriculture under document numbers 184, 01.044, 01.021/2002, and 01.021/2003.

Animals and Treatments

Nelore-cross bulls were maintained on two separate ranches, in Mato Grosso, Brazil. Bulls were approximately 2 yr of age at the initiation of the study (d 0) and grazed *Brachiaria brizantha* pastures. In study I, 72 bulls (average BW = 329 ± 4.3 kg) from Fazenda Esperança ranch were randomly assigned to one of three treatments. An intact control group (n = 24) received no treatment for the duration of the study, while a second group of bulls (n = 23) were surgically castrated 141-d after initiation of the study (d 0). The remaining bulls (n = 25) were immunized against LHRH fusion proteins. An LHRH fusion protein cocktail containing an equal molar mixture of ovalbumin-LHRH-7 and thioredoxin LHRH-7 proteins was used in each study. Treated bulls received 1.5 mg of protein in a single subcutaneous injection in the neck. Immunizations were administered as a primary injection and two booster injections on d 0, 141, and 287, respectively. Surgical castration was performed at the time of the first booster injection (d 141). Body weight and scrotal circumference were collected on d 0, 141, 287 and 385. Mean epididymidal and testes weights were collected at the time of slaughter (d 387).

In study II, 216 bulls (average BW = 258 ± 2.9 kg) from Fazenda Colorado ranch were randomly assigned to one of three treatments: intact controls (n = 72); castrate (n = 72); and immunized (n = 72). However, as a result of an exceptionally dry season, rancher II held his bulls for an extra year consequently a fourth treatment group was implemented as an additional booster was given at random to half of the immunized bulls (d 639, 4^{th} immunization). The remaining bulls that did not receive this immunization were allowed 15 months from the time of

the third immunization until slaughter. Body weight and scrotal circumference were collected on d 0, 141, 287, 639 and 741. Paired epididymidal and testes weights were collected at the time of slaughter (d 743). Both studies were designed to allow for slaughter after a withdrawal period of at least 90-d from the time of the last booster injection.

Blood samples for study I were collected (coccygeal venipuncture) on d 141, 287, and 387. For study II, blood was collected on d 141, 287, 639 and at slaughter (d 743). A centrifuge was not available at either ranch therefore, blood samples were allowed to set for 24-hr and serum was decanted into plastic vials and stored at –20° C until analysis. Serum samples were treated with 0.2% citric acid to lower pH and inactivate any potential Foot and Mouth virus, and shipped to Washington State University under APHIS permit number 50322.

Hormone Analysis

Serum testosterone, and LHRH antibody binding values were evaluated as an indication of vaccine efficacy. Serum testosterone was quantified by solid-phase RIA with a commercial kit from Diagnostic Systems Laboratory (Webster, TX). The average within-assay CV was 14.4% with a between assay CV of 8.9%. Percentage of ^{125}I-LHRH bound for each sample was quantified at a 1:1000 dilution with procedures described by Johnson et al. (1988). All serum samples and tubes brought into the United States were destroyed by incineration after assay analysis in fulfillment of APHIS regulations.

Statistical Analysis

Percent LHRH antibody binding, testosterone, body weight and scrotal circumference were analyzed using a Proc Mixed repeated measures of SAS (SAS Inst. Inc., Cary, NC). The

model included treatment and was tested using animal within treatment as the error term. Day and treatment x day interaction was tested using the residual mean square. When a significant treatment x day interaction was detected, treatments were examined within day. Orthogonal contrasts were used to compare intact to the average of castrated and immunized animals for testosterone and body weight. Comparisons for scrotal circumference were between immunized and intact bulls. Orthogonal contrast compared immunized to the average of intact and castrated animals for percent LHRH antibody binding. These comparisons were chosen prior to the study and compared the groups with the same expected biological response for specific traits. Paired epididymidal and testes weights were subjected to a one-way completely random design. Least square means are reported.

Results

Study I

Body Weight. A treatment x day interaction was detected for body weight ($P = 0.01$). On the first day of the study (d 0), the 2 yr old bulls weighed 329 ± 4.3 kg. Body weight among the three treatment groups were similar ($P = 0.71$) 141-d after initiation of the study. However, by d 287 body weight was greater ($P = 0.02$) for intact controls (472 ± 7.4 kg) than castrated and LHRH immunized animals (451 and 452 ± 7.7 kg, respectively). Two days prior to slaughter (d 385) intact bulls continued to be heavier ($P < 0.01$) at 523 ± 7.4 kg compared with 485 ± 7.6 kg and 486 ± 7.3 kg for castrate and immunized animals.

Reproductive Measurements

Scrotal circumference was similar between intact and LHRH immunized bulls on d 0 ($P = 0.74$), however, by d 141 differences in scrotal circumference were approaching significance ($P = 0.06$) for intact and immunized groups (Figure 1). Intact bulls demonstrated a steady increase in scrotal circumference throughout the study. Conversely, scrotal circumference continued to decline for the immunized group on d 287, and 385 ($P < 0.01$). Paired epididymidal and testes weights were measured at the time of slaughter an as indicator of vaccine effectiveness (d 387; Table 3). Epididymidal weights for intact bulls averaged 47 ± 1.1 g compared to 25 ± 1.0 g for the immunized group. Similarly, testes weights for the intact bulls were greater ($P < 0.01$) than that of the LHRH immunized bulls.

LHRH Antibody Binding and Serum Testosterone

A treatment x day interaction ($P = 0.01$) necessitated examination of LHRH immunization effects on percent LHRH antibody bound by day. Antibodies to LHRH were detected by d 141 in immunized bulls ($P < 0.01$; Figure 1). Antibody titer, as evaluated by ^{125}I-LHRH specific binding from serum LHRH antibodies, increased steadily and was elevated at all time points ($P < 0.01$) for immunized animals compared with castrated and intact bulls. As expected, castrate and intact animals had undetectable levels of LHRH antibody binding at all times throughout the study. Immunized animals, which received their final booster injection 100 d prior to slaughter (d 287), had mean LHRH antibody titers of 31 ± 1.8 % on d 387.

Repeated measures analysis revealed a treatment x day interaction ($P < 0.01$); therefore castration method on testosterone concentration was examined within day (Table 2). At the time of the first booster immunization (d 141), serum concentrations of testosterone were greater for

intact bulls than immunized animals ($P < 0.01$). Serum testosterone was markedly decreased in the group that was castrated after d 141 measurements were collected. Following the third immunization (d 287), mean testosterone concentrations were further reduced for immunized animals, and did not differ from castrated animals ($P = 0.87$). Immunized animals continued to demonstrated castrate concentrations of serum testosterone at the time of slaughter (d 387).

Study II

Body Weight. A treatment x day interaction was detected for body weight ($P < 0.01$). On d 0, the average bull body weight was 258 ± 2.9 kg. As in study I, body weights were similar ($P = 0.33$) 141-d after initiation of the study. However, body weight on d 287 was greater ($P < 0.01$) for intact controls (367 ± 4.7 kg) than castrated and LHRH immunized animals (346 and 343 ± 6.1 kg, respectively). By d 639, when the fourth treatment group was implemented, the intact bulls weighed 514 ± 5.2 kg compared with 456 ± 4.9 kg for castrated animals, 454 and 458 (± 7.3 kg) for immunized and 4th immunization bulls, respectively. Two days prior to slaughter (d 741) intact bulls continued to be heavier compared with castrate and both immunized groups ($P < 0.01$).

Reproductive Measurements

Scrotal circumference was similar between intact and LHRH immunized bulls on d 0 ($P = 0.90$), however by d 141 a decrease was detected in scrotal circumference compared to intact bulls and immunized bulls ($P < 0.01$; Figure 2). Intact bulls demonstrated a steady increase in scrotal circumference throughout the study. A continual decline in scrotal circumference was observed for both immunized groups with the 4th immunization animals

having an even further reduction in scrotal circumference following the last booster injection on d 639. At the end of the study (d 741), scrotal circumference was 35.4 ± 0.47 cm for intact bulls and 23.0 ± 0.61 and 19.9 ± 0.64 cm for the immunized and 4th immunization groups, respectively. As in study 1, paired epididymidal and testes weights were collected at the time of slaughter (d 743). Epididymidal and testes weights of intact bulls were heavier ($P < 0.01$) than that of both LHRH immunized groups. However, bulls immunized with three injections had larger epididymidal and testes weights than those that received the fourth immunization and were still significantly less than intact bulls.

LHRH Antibody Binding and Serum Testosterone

A treatment x day interaction ($P < 0.01$) was detected for LHRH antibody binding. LHRH antibody titers in immunized bulls followed a similar profile as those seen for immunized bulls in study I (Figure 2). Antibody titers were detected by d 141 in immunized bulls and the percent binding increased progressively throughout the duration of the study. Castrated and intact animals did not display antibody titers at any point during the 741-d study. At the time the fourth treatment group was implemented (d 639), LHRH antibody binding was 29.6 ± 2.0 % for immunized bulls and 28.5 ± 2.1 % for 4th immunization bulls (P > 0. 72). LHRH titers increased on d 741 for bulls receiving the fourth immunization in response to the d 639 booster compared to immunized bulls that did not receive the additional injection (39.4 ± 2.2 and 24.0 ± 2.2 %, respectively).

A treatment x day interaction ($P < 0.01$) was detected for serum testosterone concentrations; consequently castration method on serum testosterone was examined within day (Table 2). Testosterone concentrations were similar among the treatment groups on d 141 (P >

0.1). However, following surgical castration and at the time of the second booster injection (d 287) intact bulls had higher concentrations of testosterone compared to the other castration groups ($P < 0.01$). Intact bulls continued to demonstrate higher concentrations of circulating testosterone for the duration of the study. Castrate levels of testosterone, which did not vary, were measured for immunized bulls at all times from d 287 to d 741 ($P > 0.1$).

Discussion

The results of the present studies indicate that the LHRH-7 fusion proteins are effective in inducing an immunogenic effect in *Bos inducus* cattle at 2-yr of age. In both studies, immunization against LHRH resulted in immunocastrated bulls producing antibodies against LHRH sufficient to disrupt normal functions of the hypothalmo-pituitary-gonadal axis and alter sexual behavior (Jago et al., 1997). Though it is difficult to compare antibody binding between studies, immunized bulls had antibody titers (31.7 and 24 % binding for study I and II, respectively) at the end of each study that exceeded the classification of a "good" response described by Adams et al. (1996), where "good" was designated as > 10 % ^{125}I-LHRH bound by a 1:1,000 serum dilution. Sexually mature 3 yr old bulls immunized against LHRH showed a regression in testis size and suppression of testosterone concentrations typical of steers. Persistent LHRH antibody titers are observed in bulls that experienced a 15 mo interval between the time of the last booster injection and slaughter. In early studies by Robertson et al. (1982) bull calves immunized against conjugated LHRH showed suppression of gonadal function and sexual behavior that was reversed after only 6 mo. However, the results of study II are more comparable to those seen in the long-term suppression of testicular function in 20-25% of ram

lambs immunized at an early age (Brown et al., 1994), and 16-35% of sexually mature *Bos inducus* bulls (D'Occhio et al., 2001). In study II, 72% of bulls receiving a final booster injection 15 months before slaughter continued to exhibit LHRH antibody titers at levels that resulted in sustained low scrotal circumferences below 29 cm. Additionally, 9 % of these animals demonstrated no detectable LHRH antibody binding and also had scrotal circumferences below 29 cm. These animals are not expected to return to normal levels and may have developed non-reversible lesions in the median eminance. This type of lesion has been demonstrated in boars that were the highest responding to an immunization against LHRH (Molenaar et al., 1993).

Animal growth and development is influenced in large part by the gonadal steroids. Removal of testicular function by castration or immunization against LHRH is associated with a marked reduction in growth rate in both studies. By 287-d after initiation of the study, intact bulls had begun to out gain both surgically and immunocastrated groups. Intact bulls continued to demonstrate a superior growth compared to the castrate groups for the duration of each study. The first two weigh days (d 0 and 141) corresponded with the dry season in Brazil, and may be the reason for the similar body weights among the treatment groups. The paralleled growth rate seen in the castrated and LHRH immunized animals are similar to those previously described in unimplanted steers and immunized bulls (Huxsoll et al., 1998) and suggests the anabolic effects of testosterone are eliminated in LHRH immunocastrated bulls. An intermediate effect of weight gain and growth as demonstrated by Aïssat et al. (2002) would be expected in animals that are castrated at a young age and then compared to bulls immunized at 2 yr of age as in these studies. Interestingly the longer the time of vaccination, the bigger the differences in body weight at slaughter. In study I, when LHRH immunizations occurred over one years time a 38 kg

difference in body weight was observed when comparing intact and both castrate groups. However, when LHRH immunization occurred over 2 years time, the difference in body weight between the groups was almost double at 66 kg. Carcass performance data had been previously published on a representative group of bulls from study I (de A. Ribeiro et al., 2004), demonstrating that immunized bulls were comparable to surgically castrated steers showing increased marbling, back fat, and a decreased ribeye area compared with the intact bulls.

Decreased scrotal circumference and testes weights in LHRH immunized animals when compared to the intact bulls are consistent with those reported in beef bulls (Huxsoll et al., 1998; Cook et al., 2000) and ram lambs (Kiyma et al., 2000). These data support the early findings that LHRH immunocastration does suppress reproductive function as demonstrated by atrophy of the gonads (Arimura et al., 1973). Adams et al. (1996) suggested that 7 mo of age may be the optimal age for immunization against LHRH for highest antibody production and the most pronounced suppression of testicular weight in *Bos taurus* bulls when slaughtered 11 mo after only one primary immunization alone. However, in both studies herein 92 and 93 % of bulls receiving a booster injection 3 mo before slaughter maintained castrate like effects on reproduction. These data suggest that slaughter occurring 15 mo after the final booster injection is not sufficient in suppressing reproduction in bulls, however, the last booster injection can be delivered up to 90 d prior to slaughter.

Implications

Starting immunization against LHRH fusion proteins in two-year-old *Bos indicus* bulls is effective in producing growth and reproductive hormone profiles similar to that demonstrated by surgically castrated two-year-old bulls. Inducing a castrate like effect in immunized animals at

two years of age allows producers to take advantage of the endogenous testosterone anabolic effects during the first two years of life followed by desired steer characteristics during the third year without the associated trauma and possible death of surgical castration. The LHRH vaccine appears to be an effective alternative to traditional castration methods of 2-year-old grazing bulls in Brazil. The price spread on a steer and bull carcass in Brazil is in favor of the steer carcass but this spread is only a couple of cents (US) per kg. The Brazilian system does give a premium to the steer but does not give a large discount to bull carcass as is the case in the USA. The Brazilian grass fattened bull is an ideal production system for the use of LHRH immunization.

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Table 1. Body weight (kg) of LHRH immunized, castrated or intact bulls on pasture in Brazil

	Treatments				
	Intact	Immunized	4th[a] Immunization	Castrated	SE[b]
Day of Study I	n = 24	n = 25		n = 23	
0	325	329		334	7.6
141	357	358		363	7.8
287	472	452*		451*	7.8
385	523	486**		485**	7.6
Day of Study II	n = 72	n = 42	n =30	n = 72	
0	260	256		260	5.9
141	255	247		253	6.0
287	367	341**		346**	6.1
385	427	393**		406**	6.1
639	513	455**	458	456**	6.8
741	579	513**	509	514**	6.8

[a]LHRH late immunized animals weights are only reported on d 639 and 741 as this was the time when they were separated from immunized animals.

[b]SE = largest standard error.

*Treatment means differ from control intact animal values ($P < 0.05$).

**Treatment means differ from control intact animal values ($P < 0.01$).

Table 2. Serum testosterone (ng/mL) concentrations of LHRH immunized, castrated or intact bulls during a 385 or 741-d study

	Treatment				
	Intact	Immunized	4th[b] Immunization	Castrated	SE[c]
Day of Study I[a]	n = 24	n = 25		n = 23	
141	6.1	2.5		8.6	1.1
287	7.6**	0.3		0	1.0
385	3.2**	0.2		0	1.1
Day of Study II	n = 72	n = 72	n = 30	n = 42	
141	1.4	0.8		1.1	0.8
287	2.5**	0.7		0	0.8
385	___	___		___	
639	9.9**	1.9	1.2	0	0.8
741	2.2**	0.3	0.05	0	0.8

[a]Serum was not collected on d 0 for either study or on d 385 for study II.

[b]Testosterone values for immunized animals are reported on d 639 and 741 as this was the time at which the 4th immunization was administered and the group separated from immunized bulls.

[c]SE = largest standard error.

**Column values differ $P < 0.01$.

Table 3. Paired testes and epididymidal weights (mean ± largest standard error) for intact and LHRH immunized bulls at slaughter on d 387 for study I and day 741 for study II [a]

	Testes (g)	Epididymides (g)
Study I		
Intact	497 ± 11.5[a]	47 ± 1.0[a]
Immunized	172 ± 10.7[b]	25 ± 1.1[b]
Study II		
Intact	500 ± 6.7 [a]	60 ± 0.6[a]
Immunized	174 ± 9.0[b]	26 ± 0.8[b]
Late Immunized	80 ± 9.4[c]	20 ± 0.8[c]

[a]Column values differ ($P < 0.01$).

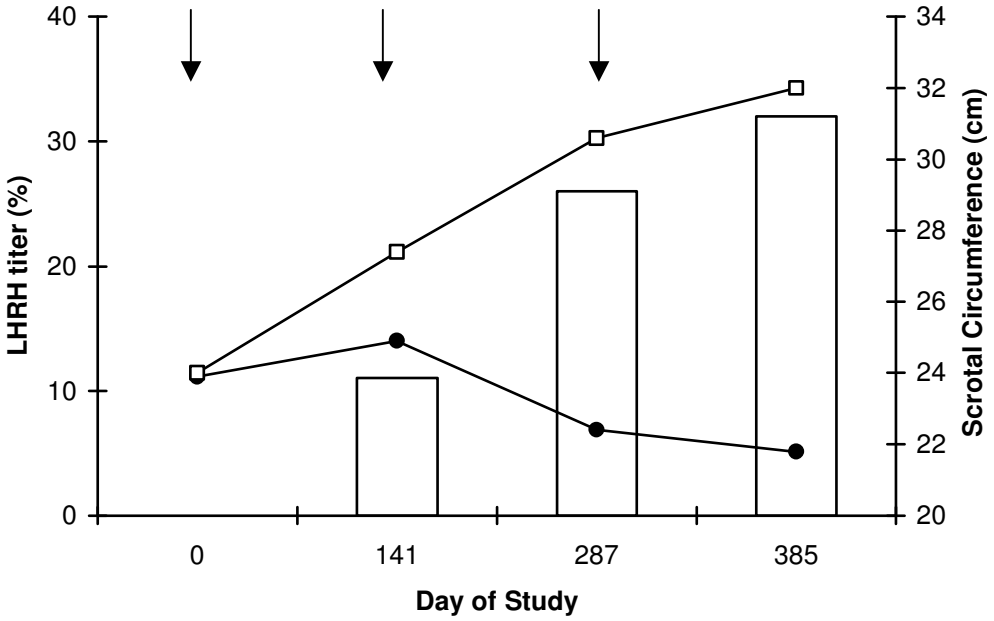

Figure 1. Line graph and right axis represents scrotal circumference of intact or LHRH immunized bulls during a 385-d study in which animals were intact (-□-), LHRH immunized (-•-). Bars indicate LHRH antibody titers as represented by % ^{125}I-LHRH binding at 1:1000 serum dilution. Arrows represent LHRH immunizations.

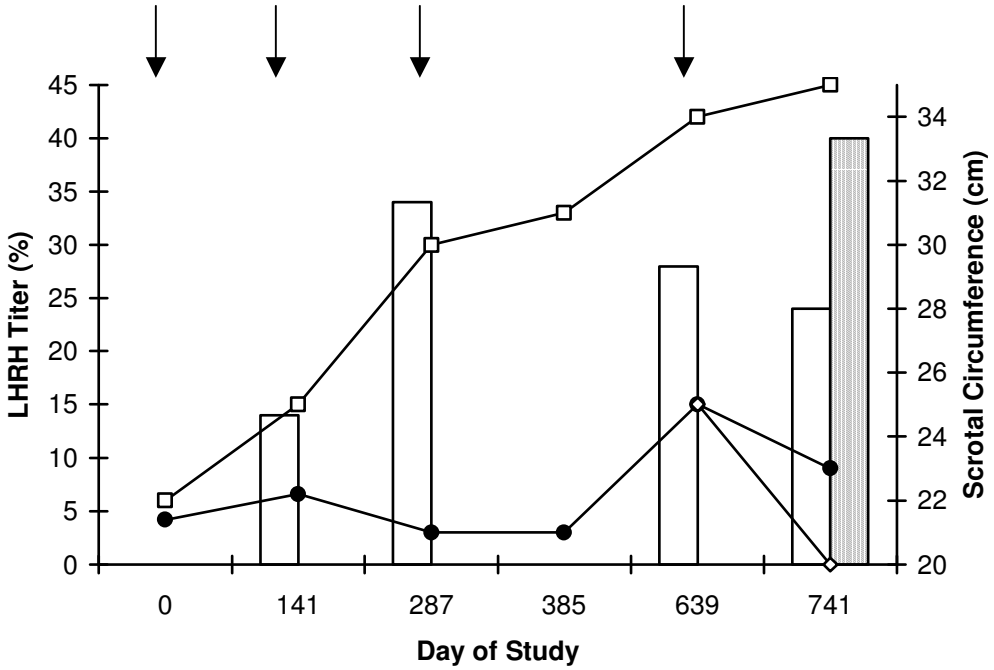

Figure 2. Line graph and right axis represents scrotal circumference of intact or LHRH immunized bulls in study II during a 741-d study in which animals were intact (-□-), LHRH immunized (-•-) or LHRH late immunized (-◊-). Bar graph and left axis indicate LHRH antibody titers as represented by % ^{125}I-LHRH binding at 1:1000 serum dilution. Arrows indicate LHRH immunizations.

CHAPTER THREE

Growth and Carcass Characteristic of Pasture Fed LHRH Immunocastrated, Castrated and Intact *Bos indicus* Bulls

Growth and carcass characteristics of pasture fed LHRH immunocastrated, castrated and intact *Bos indicus* bulls

Abstract

The effectiveness of a luteinizing hormone-releasing hormone (LHRH) fusion protein vaccine or surgical castration, at 2 yrs of age, on growth and carcass characteristics of *Bos indicus* bulls was evaluated. Seventy Nelore-cross bulls were divided into three groups: 1) immunized, 2) castrated and 3) intact control. At slaughter (3 yrs of age), intact bulls had higher body weights, ADG, carcass weights, and muscle percentage compared to immunized and surgically castrated animals. Both castrated and immunized animals had greater marbling and percent carcass fat than the intact bulls. Average tenderness scores were inferior for intact bulls compared to immunized and castrated animals, but these differences were not significant ($P > 0.05$). Juiciness, flavor, thawing, nor cooking losses differed significantly among the three groups. Immunocastration was effective in producing carcass traits similar to that of surgical castration. Therefore, immunization with LHRH fusion proteins appears to have practical utility in the management and castration of grazing bulls.

Keywords: Beef cattle; *Bos indicus*; Immunocastration; Nelore; Testis.

1. Introduction

Castration is an important tool in beef cattle management to improve meat quality, reduce aggressive behavior, and improve management ease. Along with the benefits of castration, numerous disadvantages are apparent. In addition to being labor intensive, surgical castration has the potential to cause morbidity and mortality (Bonneau & Enright, 1995) and has been associated with reduced growth rate (Huxsoll, Price, & Adams, 1998). To avoid the stress and potential death loss associated with castration, some cattle producers in countries such as Brazil do not castrate their animals. However, intact postpubertal bulls are difficult to manage, and as a consequence of their increased activity level have a higher tendency to yield dark cutting meat at slaughter (Gregory & Ford, 1983; Kempster & Lowe, 1993). Even in young animals differences in lean characteristics between intact bulls and steers can be detected. Morgan, Wheeler, Koohmaraie, Savell, and Crouse (1993) observed leaner carcasses and lower quality grades in 12 mo old bulls compared to steers of the same age. Meat from bulls also had higher shear force value. Similarly, intact bulls are leaner and produce higher yielding carcasses than steers (Field, 1971; Seidman, Cross, Oltjen, & Schanbacher, 1982). In spite of the possible problems related to lean quality of intact bulls, late castration has been recommended as a means to take advantage of greater weight gain and feed efficiency observed in bulls (Knight, Cosgrove, Lambert, & Death, 1999). Restle, Grassi, and Feijo (1994a,b) observed that steers castrated at 12 mo of age had carcass weights that did not differ from intact bulls. Conversely, these late castrated steers had greater weight gain, were heavier at slaughter, and presented heavier carcasses than steers castrated at 1.5 mo of age. As expected, castrated animals had greater fat thickness than bulls.

Generally *Bos indicus* cattle are more stressed than the *Bos taurus* cattle (Hearnshaw & Morris, 1984), and when raised in open pastures with little management, castration can be difficult and lead to death loss. Immunization against LHRH has been proposed as an alternative method to surgical castration, which allows for both improved growth rates and desired meat quality (Huxsoll et al., 1998; Cook, Popp, Kastelic, Robbins, & Harland, 2000; Aissat, Sosa, de Avila, Bertrand, & Reeves, 2002). This experiment was conducted to examine the effects of late, 24 mo, surgical and immunological castration on the weight gain, carcass production, and lean quality of *Bos indicus* bulls ranged in Brazil and slaughtered at 3 years of age.

2. Material and methods

2.1. Animals and treatments

Seventy Nelore-cross bulls, approximately 24 mo of age (average BW = 327 kg) were randomly allotted to one of three treatment groups: 1) immunized (n = 24); 2) castrated (n = 23); and 3) intact bulls (n = 23). All animals were maintained on *Brachiaria brizantha* pasture in the state of Mato Grosso, Brazil for the duration of the study. Immunized animals received a primary immunization and two booster injections administered on d 0 (2 yrs of age), 141 and 287, respectively. Immunizations were given as a single subcutaneous injection in the neck. Surgical castration was performed using traditional castration methods on d 141 of the study. Body weights were collected at each of the treatment days and two days before slaughter (d 385).

2.2. Preparation of antigen and immunization

Ovalbumin-LHRH and thioredoxin-LHRH fusion proteins were prepared using recombinant DNA technology as described by Zhang et al. (1999) and Quesnell et al. (2000). Both LHRH fusion proteins contain seven LHRH sequences inserted at different positions. LHRH fusion proteins have a 6-histidine sequence (His-tag, Novagen, 1994) at the carboxyl terminus to allow for purification by affinity chromatography. Purified ovalbumin-LHRH-7 and thioredoxin LHRH-7 proteins were combined to yield a total of 1.5 mg of protein. The LHRH fusion proteins were emulsified in modified complete Freund's adjuvant (CalBiochem, San Diego, CA), containing *Mycobacterium butyricum* for the first immunization and incomplete Freund's adjuvant for the subsequent boosters.

2.3. Carcass data

The study was designed to allow for slaughter after a withdrawal period of at least 90-d from the time of the last booster injection. All animals were slaughtered on d 387 at a commercial abattoir approximately 400 km from the ranch after a 24 hr fasting period. Dressing percentage was calculated using the d 385 live weight and the hot carcass weight. Additional carcass measurements were collected after a 24 hr chilling period at 1º C. Carcass length was designated as the distance between the anterior edge of the medial portion of the first rib to the anterior edge of the aitch bone. Leg length was measured as the distance between the aitch bone and the tarsus joint, and the arm length was measured as the distance between the olecranon and the carpus joint. Cushion thickness was measured with the help of a compass, and the arm perimeter was taken with a measuring-tape at the medium portion of the arm.

Ribeye area was measured using a ribeye area dot grid, in square centimeters, at the *Longissimus* muscle between the 12th and 13th rib. External fat thickness was measured over this muscle at this same place, at a point ¾ of the length of the ribeye from its chine-bone end. Marbling was evaluated in this muscle at this same position and was classified in six major categories (abundant, moderate, average, small, light, trace), each divided in three parts (plus, average, minus). Values for the categories were 1, 2 and 3 for trace, 4, 5 and 6 for light, 7, 8 and 9 for small, 10, 11 and 12 for average, 13, 14 and 15 for moderate, and 16, 17 and 18 for abundant. Color was also evaluated in this muscle, being classified as very dark red (1), dark red (2), slightly dark red (3), red (4), and light red (5).

Bone, muscle and fat percentages in the carcass were evaluated by the method of Hankins and Howe (1946) and adapted by Muller, Maxon, and Palmer (1973) for a section of the loin corresponding to the 9th, 10th and 11th ribs. The longissimus muscle from this section was frozen. Steaks of 1.5 cm thick were cut, thawed, and roasted in an electric grill at an internal temperature of 76 °C for 4 min each side. A trained panel evaluated tenderness, juiciness, and flavor. These three traits were determined on a scale from 1 to 9, where 1 indicated tough, extremely dry meat with undesirable flavor, and 9 was tender, extremely juicy, and flavorful. Tenderness was also evaluated by a texturometer, TA-XT2i Texture Analyzer (Stable Micro Systems), which measured the Newton's force necessary to shear the fiber.

2.4. Statistical analysis

Data were analyzed by analysis of variance for a completely randomized design using a fixed linear model with the GLM procedure of SAS (SAS, 1994). The model included the effect of

treatment. Mean comparisons, when effect was significant ($P > 0.05$), were done by t test, option PDIFF.

3. Results and discussion

3.1. Weights

Means for weights and weight gains, according to treatment, are presented in Table 1. Prior to immunization on d 0, bull weights were similar at 328 kg. Likewise, body weights (BW) were similar on d 141 at the time of the first booster injection and surgical castration. However, by d 287 there was a tendency ($P = 0.08$) for immunized and intact groups to be heavier than the castrated animals. Two days before slaughter, BW for the intact bulls was 517 kg compared with 485 and 478 kg for immunized and castrated animals ($P < 0.05$). Although average daily gain (ADG) throughout the study (d 0 to d 385) was similar for the castrated (0.39 kg/d) and immunized (0.40 kg/d) animals, both groups gained at a rate less than that of the intact bulls (0.51 kg/d; $P < 0.05$). From d 141 to d 385, the average daily weight gains were 37.7 and 31.0% greater for intact than castrated and LHRH immunized animals, respectively. Greater weight gains for intact bulls compared to steers agree with previous studies (Restle et al., 1994b; Knight et al., 1999).

3.2. Carcass composition

Means for carcass weight and composition are presented in Table 2. There were no differences in dressing percentage among the three groups; the heaviest group (intact bulls) also had the heaviest carcasses. Immunized and castrated animals had similar weights as well as

muscle and fat percentages. In contrast, intact bulls had a greater percentage of muscle and lower percentage of carcass fat than either castrated group, which is consistent with previous reports (Field, 1971; Seideman et al., 1982; Morgan et al., 1993; Restle, Grassi, & Feijo, 1996). Dressing percentage was lower (average = 50.8%) than the 60% commonly observed in American literature (Adams, Daley, Adams, & Sakurai, 1993; Morgan et al., 1993; Pringle, Williams, Lamb, Johnson, & West, 1997). This can be partially explained by the low percentage of fat and the low external fat (Table 3) on the carcasses. Euclides Filho, Euclides, Figueiredo, and Oliveira (1997) noted a greater dressing percentage for Nelore bulls, slaughtered at 440 kg that possessed a higher carcass fat thickness and fat percentage. Additionally, Brazilian abattoirs remove all excess fat including kidney, heart and pelvic fat. Dressing percentages for pasture-terminated animals observed in Brazilian literature varies from 50.3 to 56.8% (Restle et al., 1994a; Euclides Filho, 1997; Razook, Figueiredo, Ruggieri, Nardon, & Cyrillo, 2002). The three treatments presented similar percentage of bone in the carcass. The proportion of muscle to bone and edible portion to bone were also similar among the treatments. Means for quantitative aspects of the carcasses are presented in Table 3. Ribeye area (REA) is a trait that is positively correlated to the total amount of muscle in the carcass or cutability. Therefore, as the intact bulls presented a greater percentage of muscle in the carcass, they also tended ($P = 0.07$) to present a greater REA. The average for REA in immunized bulls was slightly higher than in the castrated animals. These results agree with those of Adams et al. (1993), who observed larger REA for bulls, an intermediate REA for immunized animals and smaller REA for steers. Fat thickness, for intact bulls was lower compared to that of both the castrated and immunized animals. Restle et al. (1996) also found greater ribeye area and arm perimeter and thinner fat thickness in bulls than in steers. The fat thickness of the intact bulls (2.9 mm) was less than the 3.0 mm required by the

buyers in Brazil. In this case carcasses are penalized, resulting in a lower price for the carcass. External fat is important for protection from carcass dehydration and darkening during chilling. Our results agree with Razook et al. (2002), which observed ribeye areas of 65.6 and 71.7 cm^2, and fat thickness of 4.2 and 2.9 mm in Nelore bulls slaughtered 446 and 544 kg, respectively. The greater arm perimeter in intact bulls is most likely the result of their greater development of the forequarter. Cushion thickness can also be related to the cutability of the carcass, though our results did not show any significant difference among the treatments. Arm length, leg length and carcass length were similar among the animals from the three treatments, suggesting that all animals had similar bone development. There were no significant differences due to treatments on the percentage of carcass bone. This may be explained by the fact that the animals were 24 months old before castration and the major part of bone development had already occurred. These results are consistent with those reported by Restle et al. (1994a, 1996).

3.3. Meat quality

Means for meat quality are presented in Table 4. Meat from intact bulls tended ($P = 0.10$) to be darker than the meat from immunized and castrated animals. Brandsletter, Picard, and Geay (1998) noted that castration of males results in physiologically younger animals compared to intact animals, and that the fibers produced by steers have a beneficial effect on meat color. Although not significant, Mohan Raj, Moss, McCaughey, McLauchlan, McCaughey, and Kennedy (1991) observed that bulls (vasectomized, immunized and intact) tended to produce darker meat than steers. This may occur as a result of the increased aggressive nature and susceptibility to stress.

In the present study castrated and LHRH immunized animals had significantly higher marbling scores than did the intact bulls ($P < 0.05$). Thawing and cooking losses were similar among the treatments, which agrees with data of Restle et al. (1996) comparing intact and castrated animals. Panel evaluation did not detect differences in juiciness, flavor, or tenderness among the treatments. However, it was expected that meat from intact bulls would be tougher. Although tenderness was rated lowest for the intact bulls this number was not at a level that was significantly different from the other two treatment groups ($P = 0.12$). Shear force values by the texturometer followed a similar trend with the meat from intact bulls requiring 16% more Newtons of force ($P = 0.14$). Additionally, Cook et al. (2000) observed a shear force value 16% lower for meat from LHRH immunized animals than for intact control bulls. Numerous experiments have shown differences in tenderness values between intact bulls and castrated animals (Gregory & Ford, 1983; Morgan et al., 1993; Restle et al., 1996). Less tender meat in bulls may result from greater calpastatin activity in *Longissimus* muscle, likely causing a decrease in protein proteolysis by μ-calpain through 7 days postmortem (Morgan et al., 1993). The decrease in collagen solubility as sexual development progresses has also been suggested as a basis for less tender meat in bulls (Luchiari Filho, 2000).

In addition, it has been reported that *Bos indicus* cattle, purebred or crossbred with *Bos taurus*, have problems with tenderness (Wheeler, Cundiff, Koch, & Crouse, 1996; O'Connor, Tatum, Wulf, Green, & Smith, 1997; Pringle et al., 1997; Pringle, Harrelson, West, Williams, & Johnson, 1999). In the present study, even castrated animals could not be considered tender. The average tenderness value identified by the panel was 5.4, where the best value is 9.0. Pringle et al. (1997), testing steers of varying percentage of Brahman (0, 25, 37, 50, 75 and 100%), reported that the sensory panel tenderness score decreased linearly with increasing Brahman

breeding. Similarly, higher 24-h calpastatin activities have been seen in 3/8 *Bos indicus* cattle than in *Bos taurus* cattle (O'Connor et al., 1997). Pringle et al. (1999) also found a higher calpastatin activity in muscle for Brahman (B) cattle than in Angus (A) or in F1 B x A, resulting in tougher steaks for Brahman. High correlations between the postmortem calpastatin activity and the tenderness levels have been observed in meat from *Bos indicus* breeds (Whipple, Koohmaraie, Dikeman, Crouse, Hunt, & Klemm, 1990).

4. Conclusions

Immunization against the LHRH fusion proteins was effective in producing growth and carcass traits similar to that of surgical castration. Although intact bulls produce heavier carcasses, the packing plant grader evaluated the LHRH immunized bulls as steers due to their fat covering and carcass appearances. The immunized bulls received steer prices, which were a premium over intact bulls. The practice of allowing bulls to grow with the growth stimulation of testes until 2 years of age before castration gives greater carcass weight while allowing the premium paid for a steer carcass. Therefore, the LHRH vaccine appears to have practical utility in the management and castration of 2-year-old grazing bulls in Brazil.

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TABLE 1.
Least squares means ± S.E. of live weights and average daily gains (ADG) of bulls at different times after LHRH immunization or castration

Item	Treatment[a]		
	Castrated	Immunized	Intact
Weights, kg			
Day 0 (2 yr of age)	330 ± 5.9	330 ± 5.6	321 ± 6.6
Day 141	356 ± 6.5	359 ± 6.2	352 ± 7.2
Day 287	444 ± 7.1	460 ± 7.1	467 ± 8.2
Day 385	478[b] ± 6.8	485[b] ± 6.8	517[a] ± 7.9
ADG, kg			
Day 0 – 141	0.20 ± 0.02	0.21 ± 0.02	0.22 ± 0.03
Day 0 - 385	0.39[b] ± 0.01	0.40[b] ± 0.01	0.51[a] ± 0.01
Day 141 – Day 385	0.49[b] ± 0.02	0.51[b] ± 0.02	0.67[a] ± 0.02

[a]Animals were either immunized with a LHRH fusion protein on d 0, 141 and 287; castrated on day 141 or remained intact for the duration of the study.

Means with different letters are significant different ($P < 0.05$).

TABLE 2.
Least squares means ± S.E. of carcass composition of bulls after LHRH immunization or castration

Trait	Treatment[a]		
	Castrated	Immunized	Intact
Hot carcass weight, kg	241[b] ± 3.9	248[b] ± 3.9	262[a] ± 4.5
Dressing, %	50.5 ± 0.3	51.2 ± 0.3	50.8 ± 0.4
Muscles, %	57.6[b] ± 1.0	57.3[b] ± 0.9	62.5[a] ± 1.4
Fat, %	23.9[a] ± 1.0	25.2[a] ± 0.9	17.9[b] ± 1.4
Bones, %	18.1 ± 0.6	17.3 ± 0.6	18.8 ± 0.9
Muscles:bones	3.2 ± 0.1	3.3 ± 0.1	3.4 ± 0.2
Edible portion:bones[b]	4.5 ± 0.2	4.8 ± 0.2	4.4 ± 0.3

[a]Animals were either immunized with a LHRH fusion protein on d 0, 141 and 287; castrated on day 141 or remained intact for the duration of the study.

Means with different letters are significant different ($P < 0.05$).

[b]Edible portion = the sum of muscle and fat portions in the carcass.

TABLE 3.
Least squares means ± S.E. of carcass quantitative aspects of bulls after LHRH immunization or castration

	Treatment[a]		
Trait	Castrated	Immunized	Intact
Longissimus area, cm^2	60.0 ± 2.1	63.9 ± 2.1	67.4 ± 2.4
Fat thickness, mm	4.5 ± 0.7	5.2 ± 0.7	2.9 ± 0.8
Arm perimeter, cm	36.7b ± 0.5	37.1b ± 0.4	39.7a ± 0.5
Arm length, cm	43.8 ± 0.5	44.3 ± 0.4	44.2 ± 0.4
Cushion thickness, cm	24.2 ± 0.5	23.8 ± 0.6	24.3 ± 0.6
Leg length, cm	87.8 ± 0.8	89.0 ± 0.9	89.2 ± 0.9
Carcass length, cm	131.3 ± 2.0	133.3 ± 2.1	134.4 ± 2.1

[a]Animals were either immunized with a LHRH fusion protein on d 0, 141 and 287; castrated on day 141 or remained intact for the duration of the study.

Means with different letters are significant different ($P < 0.05$).

TABLE 4.
Least squares means ± S.E. of lean quality characteristic of bulls after LHRH immunization or castration

Trait	Treatment[a]		
	Castrated	Immunized	Intact
Color[b]	3.8 ± 0.2	3.6 ± 0.2	3.0 ± 0.3
Marbling[c]	3.5[a] ± 0.4	5.0[a] ± 0.5	2.1[b] ± 0.6
Thawing losses, %	10.0 ± 0.3	9.4 ± 0.4	8.8 ± 0.6
Cooking losses, %	28.5 ± 0.9	29.5 ± 1.0	30.9 ± 1.4
Juiciness[d]	6.9 ± 0.1	7.0 ± 0.1	6.7 ± 0.2
Flavor[d]	7.6 ± 0.07	7.7 ± 0.06	7.6 ± 0.09
Tenderness[d]	5.5 ± 0.3	5.8 ± 0.2	5.0 ± 0.3
Shear force, Newton	119.1 ± 9.1	125.0 ± 6.4	145.0 ± 10.8

[a]Animals were either immunized with a LHRH fusion protein on d 0, 141 and 287; castrated on day 141 or remained intact for the duration of the study.

Means with different letters are significant different ($P < 0.05$).

[b]Variation from 1 to 5, where 3 = slightly dark red and 4 = red.

[c]Variation from 1 to 18, where 1 to 3 = trace and 4 to 6 light.

[d]Variation from 1 to 9 , where 1 = meat extremely dry, undesirable flavor, tough; 5 = average; 9 = meat extremely juicy, flavorful and tender.

CHAPTER FOUR

Microarray Analysis Of Gene Expression In Intact, Surgically Castrated, And LHRH Immunocastrated Spargue Dawley Male Rats

Microarray Analysis Of Gene Expression In Intact, Surgically Castrated, And LHRH Immunocastrated Spargue Dawley Male Rats

Abstract

Immunoneutralization of the hypothalamic decapeptide LHRH is know to be effective in modulating the reproductive function of mammals. The means by which LHRH immunocastration exerts its effect on reproduction is considerably different from surgical castration. To characterize the changes in regulation of gene expression in both surgical and immunocastration, mRNA from anterior pituitaries was screened using Affymetrix 230A rat Genechip oligonucleotide microarrays. Fifty-one Sprague Dawley male rats were divided into four treatment groups: 1) intact; 2) surgically castrated; 3) LHRH immunocastrated; and 4) rats which received both surgical and LHRH immunocastration. All rats received a primary injection and two booster injections of the carrier protein (recombinant ovalbumin) or the recombinant ovalbumin LHRH-7 fusion protein at a dose of 50 μg/rat. At the end of the study, rats were sacrificed and anterior pituitaries were removed. RNA was purified and hybridized to the 230A rat microarray containing 16,000 rat genes. Analysis identified transcripts that were up- or down-regulated two fold change or greater. Genes previously reported to be LHRH or G-protein regulated were identified including FSHb, LHb, and Rgs4. This study has begun to identify genes that are uniquely regulated by LHRH immunocastration.

Introduction

The use of active immunization against LHRH as a mechanism by which to modulate reproduction has been investigated extensively in livestock (Falvo et al., 1986; Brown et al., 1994; Aïssat et al., 2000) and rodents (Fraser et al., 1974; Giri et al., 1990; Awoniyi et al., 1993). However, a paucity of information remains on the cellular and neural effect of immunocastration. LHRH is secreted in a pulsatile fashion from the median eminence into the hypothalmo-hypophysealportal system. Pituitary gonadotropes in the adenohypophesis express receptors specific for LHRH. Hypothalamic LHRH binds the heptahelic, G-protein coupled membrane receptor on the surface of the gonadotropes and activates intracellular signal transduction pathways to cause both synthesis and intermittent release of LH and FSH (Norwitz et al., 1999). The pattern of LHRH pulse frequency differentially regulates the glycoprotein hormones, alpha subunit (*cga*), LHβ subunit and FSHβ subunit gene expression (Haisenleder et al., 1991; Norwitz et al., 1999; Kaiser et al., 1997; Leung et al., 1987). The signal cascade resulting from the LHRH-mediated transcriptional activation of the LHRH receptor gene stimulate an increase in phosphoinositide turnover within the plasma membrane, cytoplasmic diacylglycerol levels, and the activation of protein kinase C (Stojilkovic and Catt, 1995; Norwitz et al., 1999).

Interest in the cellular aspects of LHRH active immunization arises from the knowledge that although immunocastration and surgical castration are both effective methods used to suppress reproduction, the manner by which each is achieved is unique. Following surgical castration, and the complete removal of testosterone negative feedback on the pituitary and hypothalamus, concentrations of LH and FSH increase dramatically (Schanbacher, 1982; Adams and Adams, 1992). Active immunization against LHRH results in immunoneutralization of

LHRH by binding the decapeptide before it is able to attach to its membrane receptor on the pituitary gonadotropes. This effectively suppresses the synthesis and secretion of LH and to a lesser extent FSH, subsequently the gonadal steroids (Brown et al., 1994; Aïssat et al., 2000). Furthermore, studies suggest that immunization against a peptide antigen that is dissolved in Freund's adjuvant, may lead to T cell-mediated autoimmune reactions against the antigen and unwanted autoimmune reactions directed against the hypothalamic LHRH neurons (Molenaar et al., 1993). The production site of LHRH occurs in the cell bodies in the preoptic area and hypothalamus where they are protected by the blood brain barrier from circulating macromolecules. Yet, axons of the LHRH neurons extend into the median eminence, beyond protection of the blood-brain barrier. Therefore, the objective of the present study is to characterize genes in the pituitary that respond to surgical, or immunological castration and evaluate histological changes in the hypothalamus and andenohypophysis associated with each type of castration.

Materials and Methods

Preparation of Antigen

Ovalbumin-LHRH-7 fusion protein was prepared using recombinant DNA technology as previously described (Zhang et al., 1999). Ovalbumin-LHRH-7 contains seven LHRH sequences inserted at four different positions in the ovalbumin gene fragment. The *E. coli* strain BL21(DE3) (Novagen Inc., Madison, WI) was used to express the fusion protein. The recombinant fusion protein expresses a 6-histidine sequence (His-tag, Novagen, 1994) at the carboxyl terminus to facilitate purification of the LHRH fusions proteins via nickel affinity chromatography. Fifty μg of purified recombinant ovalbumin or recombinant ovalbumin

LHRH-7 were emulsified in a water-oil adjuvant to yield a total injectable volume of 100 µl/rat. The adjuvant contained *Mycobacterium butyricum* (CalBiochem, San Diego, CA), as an immunostimulant for the primary immunization and the bacterial proteins were excluded in the adjuvant for booster injections.

Animals and Treatments

All procedures performed on rats were approved by the Washington State University Institutional Animal Care and Use Committee (protocol 1780). Sprague Dawley male rats approximately 8 wk of age at the initiation of the study were purchased from Charles River Laboratories (MA). Rats were housed two per cage in the Experimental Animal Lab Building of Washington State University. All animals had ad libitum access to food and water and were exposed to controlled climate conditions, with a 14L:10D photoperiod. Following a one-month acclimation period, 51 rats (average BW = 325 g) were randomly assigned to one of four treatment groups: (I) intact; (C) castrated; (L) LHRH immunized; and (LC) LHRH immunized and castrated. The intact control group (n = 12) and the castrate group (n =12) received 50 µg/rat of the recombinant ovalbumin protein, while rats in the LHRH immunized (n = 15) and LHRH immunized and castrate (n = 12) groups received 50 µg/rat of the recombinant ovalbumin LHRH-7. Immunizations were administered as a single subcutaneous injection at the base of the tail on day 0, 35 and 96. Surgical castration for C rats occurred on d 40 of the study. Rats were anesthetized using a ketamine (70 mg/kg), and xylazine (10 mg/kg) mixture, delivered as an intraperitoneal injection. Once completely anesthetized, the rat was laid in a lateral recumbent position and the scrotum was cleaned using a betadine solution. A 1 cm long incision was made through the skin at the tip of the scrotum. A 5 mm incision was made at the tip of the tunica

albuginea and the cauda epididymis was pulled out followed by the testis. A single suture was placed around the spermatic cord and blood vessels, and the testis was removed by cutting below this suture.

Blood samples and body weights were collected from each animal once a month for the duration of the study. Blood was collected via the saphinous vein into microcentrifuge tubes and allowed to clot at 4° C overnight, serum was then separated by centrifugation at 1,500 x g for 20 min at 4° C. Serum was decanted into plastic vials and stored at - 20° C until assayed.

Hormone Analysis

Serum testosterone, and LHRH antibody binding were evaluated as an indication of vaccine efficacy. Serum testosterone was quantified by solid-phase RIA utilizing components of a commercial kit from Diagnostic Systems Laboratory (DSL, Webster, TX). LHRH antibody titers were evaluated by percentage of ^{125}I –LHRH bound after serum was diluted 1:1000 as described by Johnson et al. (1988).

RNA Preparation

Pituitaries from nine rats in each treatment group were collected. Posterior pituitaries were dissected away and discarded, while the anterior pituitary was snap frozen on liquid N, and then stored at –80 °C. Three rat anterior pituitaries per treatment group were pooled to obtain necessary amounts of RNA. RNA was isolated using Trizol according to manufacturer's instructions (Invitrogen, Carlsbad, CA). RNA concentration and purity was determined by measuring 260/280 nm ratios.

Affymetrix GeneChip Analysis

Total cellular RNA (10 µg) was used to synthesize the microarray target. Target synthesized from RNA from two independent pools of anterior pituitary preparations for each of the four treatments was hybridized on the microarrays. The rat 230A microarray from Affymetrix was used in all hybridizations. This array contains 16,000 genes from *Rattus norvegicus.* Probes consist of 16 pairs of 25 bp oligonucleotides for each gene. A mismatch member of each pair contains a single base point mutation, the signals of the pairs are compared to assess hybridization specificity. Results were analyzed using Affymetrix Microarray Suite 5.0 (MAS) software.

Histology

All animals were sacrificed on d 208 of the study. Testes, epididymidal, prostate, and seminal vesicles were collected and weighed. In addition, testicular tissue samples were obtained from each animal and placed in fixative for histological examination (Bouin's solution, Sigma-Aldrich, St. Louis, MO). Three rat pituitaries from each treatment group were collected and preserved in 10% buffered formalin for histological examination.

Statistical analysis

Percent LHRH antibody binding, testosterone, and body weight were analyzed using Proc Mixed procedures of SAS (SAS Inst. Inc, Cary, NC). The model included treatment and was tested using animal with treatment as the error term. Day and treatment x day interaction was tested using the residual mean square. When a significant treatment x day interaction was detected, treatment effects were examined within day. Orthogonal contrasts were used to

compared treatment groups with the expected similar and different biological response for a specific trait. Reproductive weights were subjected to a one-way completely random design. Least square means are reported.

Results

Body Weight

A treatment x day interaction was detected for body weight ($P > 0.01$). No difference was detected in body weight at initiation of the study (d 0; $P = 0.47$), where average body weight was 325 ± 8.3 g. Body weight among the treatment groups remained similar on days 35, 63 ($P > 0.10$). On day 96, intact control rats began to demonstrate a greater weight than C, L and LC rats ($P = 0.06$). By day 129, intact rats weighed 586 ± 14.9g compared to 521, 520, and 532 (± 14.9) for C, L and LC, respectively. Similarly, a greater body weight for intact controls was demonstrated on d 161 and at the end of the study (d 208; $P < 0.01$), with no detectable difference among C, L or LC groups.

LHRH Antibody Binding and Serum Testosterone

Repeated measures analysis revealed a treatment by day interaction ($P = 0.01$); therefore, effect of treatment on LHRH antibody bound was examined within day. Antibody titer was evaluated by ^{125}I-LHRH specific binding to LHRH serum antibodies. LHRH antibodies were detected by d 35 in L rats (5.7 ± 1.4 %) and LC rats (6.8 ± 1.5 %). LHRH titers increased steadily from this point until the end of the study in animals receiving the ovalbumin LHRH-7 fusion protein. Rats in C and I groups also showed a slight antibody titer for the final three

collection dates (d 129, 161, and 208). By the end of the study (d 208), LHRH antibody titers for LC and L rats were 21.7 ± 4.1 % and 31.7 ± 3.9 %, respectively.

A treatment x day interaction ($P < 0.01$) for testosterone necessitated examination of testosterone concentration within day. Testosterone concentrations did differ at the beginning of the experimental period such that C and I rats had higher circulating testosterone (5.8 ± 0.8 ng/mL and 6.7 ± 0.9 ng/mL, respectively) than L or LC (3.3 ± 0.8 ng/mL and 3.0 ± 0.9 ng/mL, respectively) rats ($P < 0.01$). A marked reduction in serum testosterone concentration was noted after surgical castration. Serum testosterone was also reduced in rats that were immunized against LHRH by 35 d after primary immunization. LHRH immunized rats and C rats had similar concentrations of testosterone by d 63 ($P = 0.88$). The LHRH antibody titer required to decrease testosterone concentrations to castrate levels is approximately 15 % ^{125}I-LHRH binding (Figure 2). Intact rats continued to display a greater testosterone concentration compared to L rats for the duration of the study, but a gradual decline in testosterone was observed over the course of the study.

Reproductive Weights

Paired testis weight at the time of sacrifice was greater ($P < 0.01$) for I rats (3.6 ± 0.2 g) than L rats (0.84 ± 0.2 g). Epididymal weights of these two groups showed this same effect. Similarly, seminal vesicle weights differed by group ($P < 0.01$) and averaged 0.1, 0.1, 0.2, and 1.2 (± 0.08) g for C, LC, L and I rats. Parallel effects in prostate weights among the treatment groups were also observed.

Histology

Histological examination of testis samples collected from each sacrificed rat indicated that seminiferous tubule diameter was decreased in L rats compared to I controls. Seminiferous tubule atrophy was noted in all LHRH immunized rats. Tubule diameter for LHRH immunized rats was half that of intact controls (100 ± 0.8 μm versus 260 ± 0.7 μm; $P < 0.01$). Testes of L rats were completely devoid of spermatozoa and it appeared as if any mitotically dividing cell were undergoing apoptosis and had sloughed to the lumen of the seminiferous tubule. Conversely, full spermatogenesis was evident in control intact rats.

Gene Expression Profiling

To characterize changes in gene expression that are involved in response to immunocastration versus surgical castration, pooled anterior pituitary RNA from each of the treatments was used to synthesize targets to screen oligonucleotide microarrays. The rat 230A Affymetrix GeneChip containing 16,000 genes and EST's was used. Quantification of target hybridization allows for determination of relative amount of an individual gene in a tissue, as well as the change in expression of that gene (increase, decrease or no change) when comparing data among multiple arrays. In each array screening experiment two separate preparations of rat anterior pituitaries from each treatment group was hybridized to its own array to generate replicate data sets.

A statistical means, known as absolute analysis, by which to determine the presence or absence of a gene is provided by the analysis of the oligonucleotide microarray. This test compares the hybridization signal of the target to its complementary sequence and a mismatch sequence. Sixteen complementary probes, located throughout the array, are used to represent each gene. The intensity of the signal from these probes is used to determine the presence of a

gene and a p-value calculated from these data. A significant p-value ($P \leq 0.05$) indicates that a gene is present on the array. Additionally, a comparison analysis is conducted to determine a change in gene expression following a specific treatment. In this type of analysis, data from one microarray hybridization is used as the baseline of gene expression and compared to a microarray hybridization of another (experimental). In this regard gene expression changes are attributed to changes induced by the treatment. Filter criteria for comparison analysis were genes with signals of 100 or greater and at least a two fold change in gene expression.

Identification of genes involved in surgical and immunocastration

Absolute analysis for each treatment group is outlined in Table 1. All possible combinations of microarray comparison analysis were made among the different treatment groups for thorough analysis. After examination of the various analyses, the most biologically relevant treatment group comparisons were determined to be LC vs. C, and L vs. C. In both cases LC and L were compared using C as the baseline expression data. Pituitaries from LC rats showed a total of 99 genes that were different from the C baseline by at least two-fold. Similarly, L rat pituitaries showed 67 genes that differed from the C baseline by at least two-fold. When these comparisons were examined for genes that were similar between both groups, sixteen were identified (Table 2).

A number of changes in gene expression that correspond to signaling and immune function were identified. Follicle stimulating hormone beta (FSHβ) and luteinizing hormone beta (LHβ) were decreased in the pituitaries of L immunocastrated rats. Others include Rgs4, a regulator of G-protein signaling, and Kcnmb4, which has calcium activated potassium channel activity; these genes were also decreased.

Discussion

Oligonucleotide microarrays were utilized in this study to provide information on gene expression of the anterior pituitary gland after animals are exposed to either surgical or immunological castration. Information from these analyses can be used to further understanding of the molecular and cellular mechanisms by which immunological castration is effective. This knowledge may help researchers improve the effectiveness of the vaccine since variable response to such a vaccination has been noted (Cook et al., 2000; Kiyma et al., 2000). The overall effect in gene activity for immunocastrated rats compared to castrated rats was a downregulation of gene activity. Genes related to hormonal regulation of reproduction were downregulated following immunization against recombinant ovalbumin LHRH-7. The expression of FSHβ and LHβ were among the genes that demonstrated the highest decrease in expression following immunocastration. This was an expected effect since immunoneutralization of LHRH has been shown to suppress circulating concentrations of the pituitary gonadotropins, LH and FSH (Brown et al., 1994; Aïssat et al., 2002). Similarly, a decrease was detected for regulator of G-protein signal 4 (Rgs4). Though this gene had not previously been identified to participate in active immunization against LHRH, it's expression may not be such a surprise since Rgs4 has GTPase activity and works to return Gα to the GDP-bound inactive state (De Vries and Farquhar, 1999; Wieland and Mittmann et al., 2003). Additionally, the expression of several genes related to immune function was affected by immunocastration when compared to surgical castration. These genes include Cd74, Ifitm3I, and H2-M3. Again this might be an expected result due to the immunomodulatory effects of active immunization against LHRH. We can also deduce that this is not merely caused by the adjuvant or the recombinant ovalbumin alone since

the castrated rats also received the same amount of adjuvant and vehicle, as did the immunocastrated groups.

Immunization against LHRH had an effect on both mean weight and weight gain among the treatment groups. This is consistent with weight gains for intact bulls compared to steers (Huxsoll et al., 1998; Knight et al., 1999; de A. Ribeiro et al., 2004). Gonadal steroids are known to influence animal growth and development and removal of the testes results in a reduction in growth rate and feed efficiency in cattle (Gregory and Ford, 1983). In this study no difference in growth weight was observed in the intact controls until 129 days after the primary injection. However, for each weight collected after this point the intact controls were heavier compared to C, L or LC rats. There was also no intermediate effect of body weight since all the animals received immunocastration or surgical castration in close time proximity. These results are similar to those shown by our lab where Nelore bulls and steers that receiving immunocastration and surgical castration at the same time gained weight in a parallel manner (de A. Ribiero et al., 2004). This demonstrated that the means by which the gonadal steroids were suppressed had no effect on the resultant decrease in rat body weight.

All but two L rats responded to immunization against the recombinant ovalbumin LHRH-7 protein with high antibody titers. These two "non-responders" had low LHRH antibody titers at the end of the study and testosterone concentrations above the average for the L group. Additionally, the non-responders demonstrated testicular weight and seminiferous tubule diameter comparable to the intact controls. LHRH antibody titers were effective in downregulating the expression of LHβ and FSHβ genes and consequently suppressing serum concentrations of testosterone. The unique β subunit of the gonadotropins is encoded by a separate gene and contained on a separate chromosome than the common α subunit (Gharib et

al., 1990). The majority of LH and FSH synthesis and secretion occurs from Long-term suppression of LH and FSH, caused by immunization against LHRH, has been shown to cause testicular atrophy that subsequently leads to a decrease in testosterone concentrations and eventually disruption of spermatogenesis in various mammals (Chappel et al., 1980; Awoniyi et al., 1989; Schanbacher, 1982; Jago et al., 1997; Cook et al., 2000). The effect of immunocastration on pituitary LH and FSH are vastly different than those seen in surgically castrated animals. Gonadectomy leads an increase in LH concentrations 5 to 10 times higher than intact males (Parlow, 1964). Furthermore, this marked increase in the gonadotrophins results in the vacuolization of the multihormonal basophilic cells in the pituitary, which make up the majority (60%) of the cells responsible for LH and FSH synthesis and secretion (Shacham et al., 2001). This morphological change in these cells is referred to as castrate cells. Interestingly though is the fact that immunocastration did not elicit this same morphological pituitary change. The gonadotrophic cells for L rats was similar to that seen in I and L + C rats. Molenaar et al. (1993) also reported this lack of castration cells in LHRH immunized pigs.

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Table 1. Absolute analysis report for each of the four treatment groups

Treatment	% Present	Number of genes Present	% Absent	Number of genes Absent
Intact	56.8 ± 1.2	9088	41.1 ± 1.2	6624
Castrate	57.4 ± 1.1	9184	40.6 ± 1.1	6496
LHRH Immunized	57.4 ± 1.6	9184	40. 8 ± 1.6	6528
LHRH Immunized + castrate	58.8 ± 1.6	9404	39.3 ± 1.5	6288

Table 2. Gene expression changes in 16 genes from LHRH immunized and LHRH immunized and castrate rat pituitaries compared to castrate

Gene Symbol	**Accession No.**	**L vs C Fold Change**	**LC vs C Fold Change**
Fshb	NM_008045	- 18.5	- 8.9
Lhb	NM_008497	- 8.5	- 4.8
Rgs4	NM_009062	- 10.5	- 7.6
Lyzs	NM_017372	- 4	- 4.9
Nefl	NM_010910	- 3.1	- 3.3
Kcnmb4	NM_021452	- 4.4	- 4.2
Ostf1	NM_017375	- 2.6	- 2.9
Ii	NM_010545	- 3.6	- 3.6
Ets2	NM_011809	- 2.8	- 2.8
Hbb	AK011062	- 3.4	- 2.9
Anxa5	NM_009673	- 2.3	- 3.0
Slc17a6	NM_080853	- 2.6	- 3.3
Exosc9	NM_019393	- 2.5	- 2.7
Hba1	AA109900	- 3.0	- 2.6
SSr4	NM_009279	- 2.5	- 2.5
Ifitm3I	NM_025387	- 2.6	- 2.5
H2-M3	NM_013819	- 3.1	- 4.2

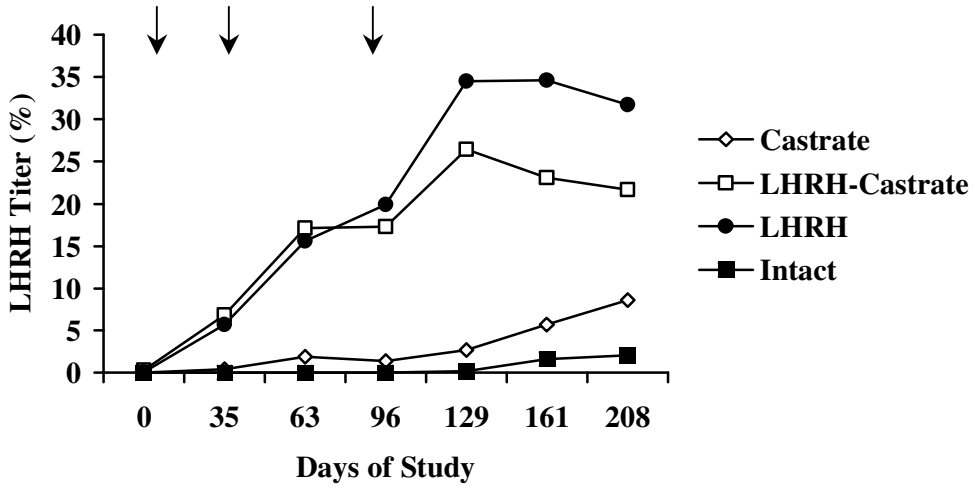

Figure 1. LHRH antibody titers of castrated (-♦-), LHRH immunized and castrated (-□-), LHRH immunized (-◊-), and intact (-■-) male rats over a 208-day study. Antibody titers are expressed as % ^{125}I-LHRH bound at a 1:1000 serum dilution. Arrows indicate when LHRH immunizations were administered.

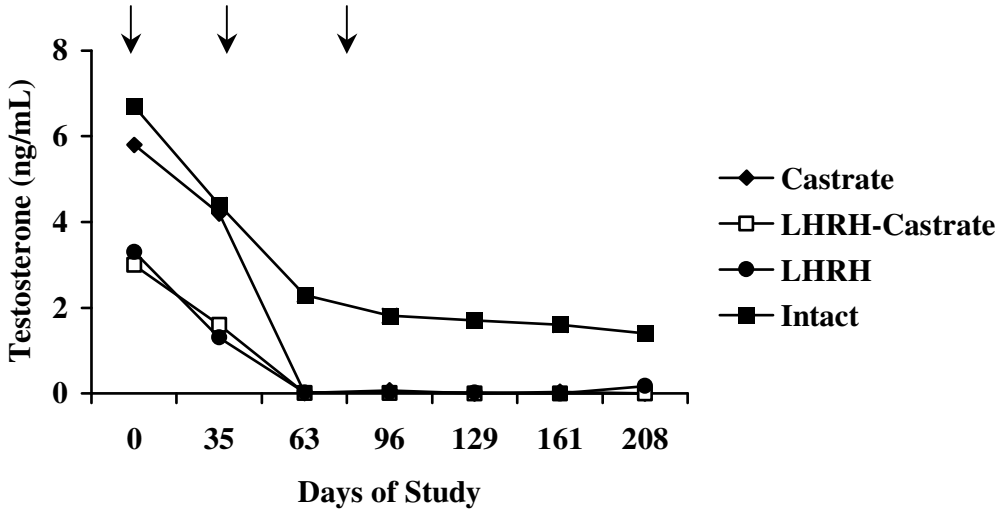

Figure 2. Serum testosterone concentrations of castrated (-♦-), LHRH immunized and castrated (-□-), LHRH immunized (-◊-), and intact (-■-) male rats over a 208-day study. Arrows indicate when LHRH immunizations were administered.

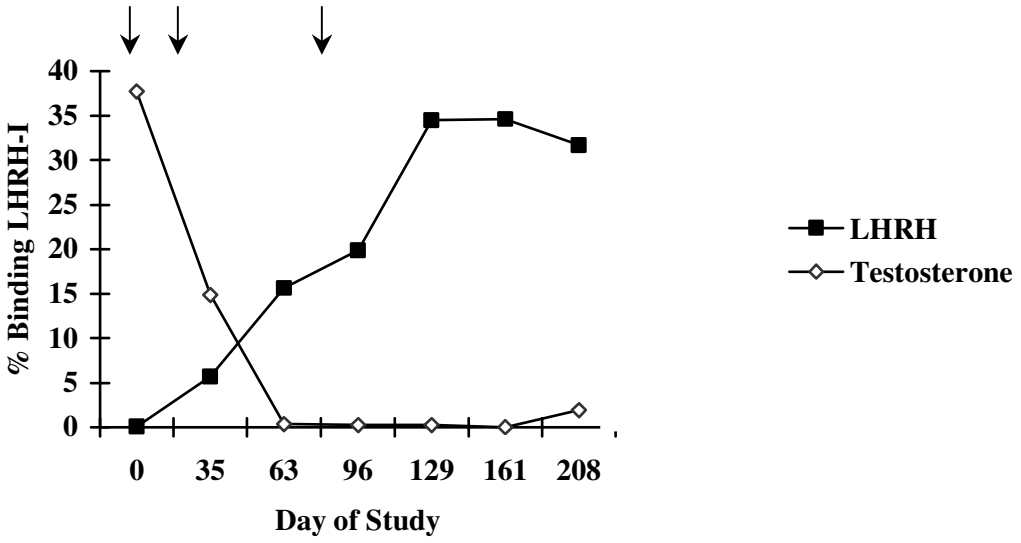

Figure 3. Relationship between serum testosterone concentrations and LHRH antibody binding for castrated (-♦-), LHRH immunized and castrated (-□-), LHRH immunized (-◊-), and intact (-■-) male rats over a 208-day study. Arrows indicate when LHRH immunizations were administered.

A **B**

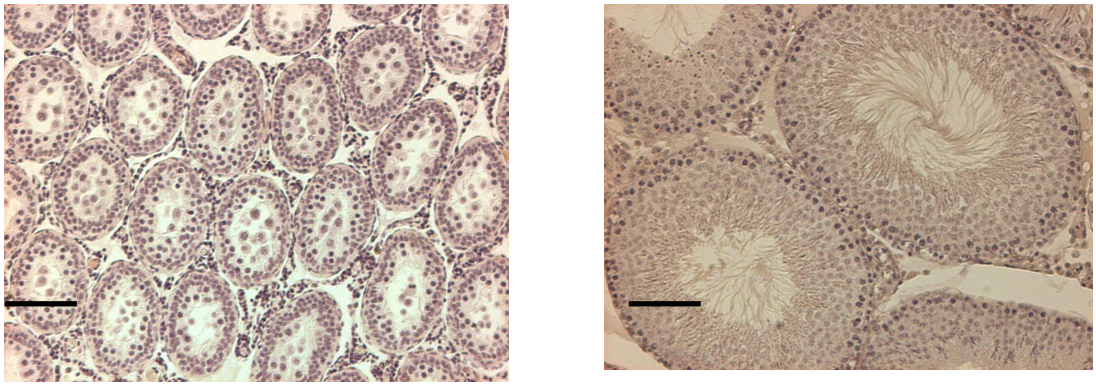

Figure 4. Photomicrographs of cross sections of A) LHRH immunized and B) Intact Sprague Dawley seminiferous tubules. 100 x magnification. Bar = 100 µm.

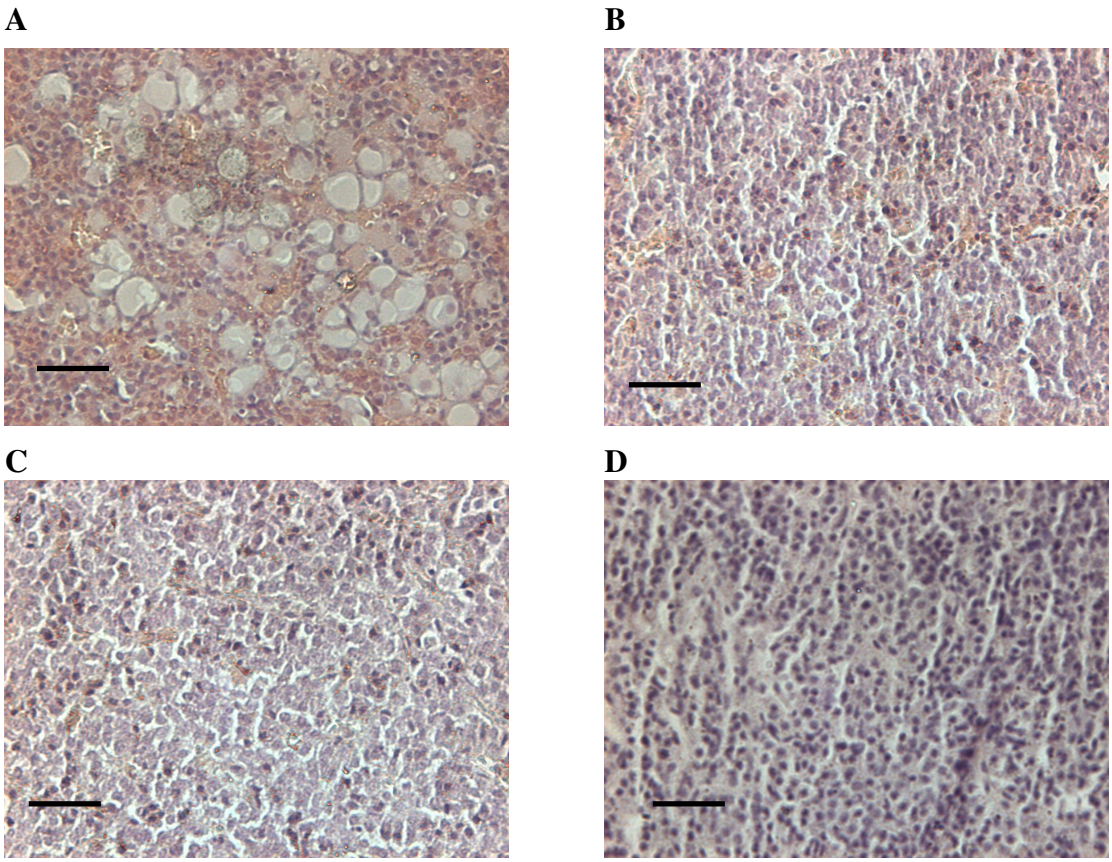

Figure 5. Photomicrograph of pituitary cross sections from A) castrated, B) LHRH immunized, C) LHRH immunized and castrated, and D) intact male Sprague Dawley rats. 100 x magnification. Bar = 100 µm. Note that the castrate cells in A are not found in LHRH immunized, LHRH immunized and castrate or intact rat pituitaries.