ACTIVE IMMUNIZATION AGAINST GONADOTROPIN-RELEASING HORMONE

an effective tool to block the fertility axis in mammals

Johan Turkstra

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ACTIEVE IMMUNISATIE TEGEN GONADOTROPINE-RELEASING HORMOON

een effectieve methode om de fertiliteitsas in zoogdieren te blokkeren

(met een samenvatting in het Nederlands)

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Chapter 1

General Introduction

1. Fertility axis regulation by GnRH

Gonadotropin-releasing hormone (GnRH), also known as luteinising-hormone releasing hormone (LHRH) is produced in neurons in the hypothalamus. It is transported via axons to small blood vessels (portal vessels) in the median eminence, where it is released into the blood. The blood vessels are draining the anterior pituitary, allowing GnRH to reach the anterior pituitary in high concentrations. In the pituitary, GnRH binds to the GnRH receptors on the gonadotropic cells to stimulate the release of follicle-stimulating hormone (FSH) and luteinising hormone (LH) to the circulation. The pulsatile secretion pattern of GnRH induces the cyclic release of LH and to a lesser extent of FSH.

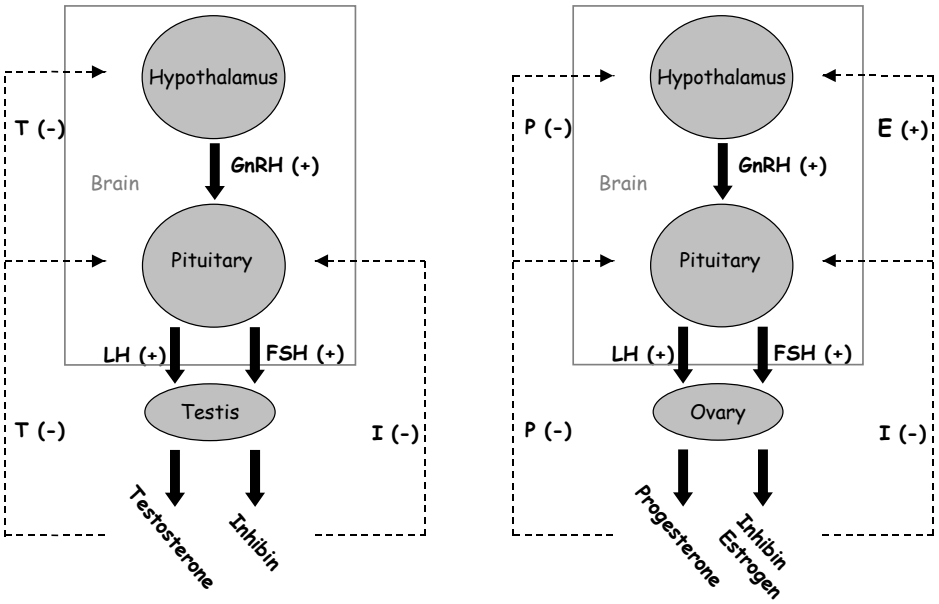

Figure 1. Schematic overview of the fertility axis in male (left) and female (right) mammals. GnRH, LH and FSH are responsible for secretion of testosterone (T), progesterone (P), estrogen (E) and inhibin (I). High levels of T, P and I, inhibit either GnRH, LH or FSH secretion via a negative feedback. High levels of estrogens are responsible for a positive feedback on GnRH at time of proestrus, subsequently leading to the LH surge and ovulation.

In male mammals, LH stimulates the synthesis and secretion of androgens (e.g. testosterone) from the Leydig cells in the testes. High levels of androgens, directly and indirectly inhibit LH secretion by a feed-back mechanism acting on the pituitary and hypothalamus, where it inhibits GnRH release. FSH is responsible for the initiation of spermatogenesis, whereas in adults it may play a role, together with testosterone, in

maintaining sperm production. FSH binds to specific receptors on the Sertoli cells to stimulate the production of many growth factors and other factors such as androgen-binding protein (ABP). ABP is necessary to maintain a high concentration of testosterone in the seminiferous tubules by binding this androgen. Inhibin, also secreted by the Sertoli cells is the major feedback regulator of FSH release. The production and secretion of inhibin is regulated by FSH.
In female mammals, FSH induces follicle growth and subsequently estradiol and inhibin secretion by the granulosa cells. High estradiol levels, produced by mature follicles, lead to a positive feedback on the hypothalamus at time of proestrus, which causes the LH surge responsible for ovulation. During this period, inhibin is secreted by the granulosa cells, causing an inhibition of FSH release. After ovulation the luteinised granulosa and the theca cells start to produce high levels of progesterone, which in turn inhibits LH and FSH secretion from the pituitary.

2. Targeting GnRH to manipulate the fertility axis

Manipulation of the fertility axis by targeting GnRH has potentials as a tool to block gonadal function in male and female mammals, in order to delay puberty, prevent sexual and aggressive behavior and sex odors, to establish infertility or to treat reproduction-related diseases. Several approaches may be appropriate to achieve this, like treatments with GnRH agonists or antagonists, passive or active immunization against GnRH or the use of GnRH targeted toxins.

2.1 GnRH agonists

GnRH is a peptide of 10 amino acids with the following amino acid sequence in 3-letter code:

pyro-Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-amide.

Soon after the discovery of GnRH, the peptide was used to treat infertility (Schally et al., 1973). Administration of low doses of GnRH in a pulsatile manner stimulates the pituitary-gonadal axis similar to endogenous GnRH. However, chronic administration at higher doses has an inhibitory effect on the pituitary-gonadal axis. Initially LH and FSH secretion are stimulated, this is the so-called flare-up, but due to down-regulation and desensitization of the GnRH receptors it eventually leads to suppression of pituitary-gonadal function. GnRH agonists with an improve half-life have been developed, leading to GnRH super agonists. Due to their inhibitory effects on the pituitary-gonadal axis, these GnRH super agonists have been used successfully for treatment of reproduction-related diseases, such as precocious puberty (Partsch and Sippell, 2002), endometriosis (Hornstein et al., 1998),

uterine fibroids (Stovall et al., 1991) and malignancies which are dependent on gonadal steroids, such as prostate cancer (Seidenfeld et al., 2000) and breast cancer (Taylor et al., 1998).
In the veterinary field, however, results are inconsistent. GnRH agonists can be used to prevent ovulation in female cattle, while in male cattle they have a stimulatory effect on gonadal activity (D'Ochhio et al., 2000). In stallions, suppression of gonadotropins was reported after a single GnRH implant (Johnson et al., 2003), while others found a stimulatory effect on sexual behavior and sperm quality (Sieme et al., 2004). In mares GnRH agonist treatment extended estrous intervals, but did not affect ovarian function (Johnson et al., 2002). In other species, agonist treatment totally suppressed gonadal activity, for instance in male and female pigs (Reid et al., 1996; Sinclair et al., 2001), and male and female dogs (Vickery et al., 1985; Vickery et al., 1989).

2.2 GnRH antagonists

The mechanism of action of GnRH antagonists is based on the permanent occupation of the GnRH receptor, which prevents endogenous GnRH to bind to its receptor (Karten and River, 1986). In contrast to agonists, GnRH antagonists do not induce an initial stimulating effect, but directly inhibit LH and FSH secretion. The therapeutic applications of GnRH antagonists are the same as those of the agonists, although the lack of a flare-up respons may be advantageous in case of short term treatment, such as in vitro fertilization or treatment of metastasized prostate tumors (Huirne and Lambalk, 2001). The GnRH antagonists Ganirelix and Cetrorelix are currently available for inhibition of premature LH surges in women undergoing ovarian hyperstimulation (Howles, 2002). The antagonist Abarelix was only conditionally approved for treatment of prostate cancer in men for whom agonist therapy may not be appropriate. Clinical trials which should lead to approval of the drug revealed, however, that Abarelix could induce immediate allergic reactions, including hypotension and unconsciousness, requiring complicated administration procedures (Harrison et al., 2004).
In animals, GnRH antagonist treatment has given variable results. In female cattle, it reduces hormone levels and causes a delay in ovulation (Rieger et al., 1989), whereas others observed only a short suppressive effect on gonadotropin levels (Ulker et al., 2001). In female pigs, the LH surge and ovulation were blocked by antagonist treatment, whereas FSH and oestradiol secretion were not affected (Brussow et al., 2001). Total suppression of testosterone and reduction in testis size were observed in young boars given a high dose of GnRH antagonist for 3 weeks (Ziecik et al., 1989). In stallions, antagonist treatment suppressed hormone levels and libido (Fortier et al., 2002; Hinojosa et al., 2001), while in mares effects were variable (Briant et al., 2002; Guillaime et al., 2002). In general,

application of GnRH antagonists in animal species is limited due to the high costs of the treatment and the inconsistent effects.

2.3 Active immunization against GnRH

Antibodies raised against synthetic GnRH peptide vaccines, neutralize endogenous GnRH. This results in infertility in both male and female mammals (Fraser, 1982). In males, inhibition of pituitary secretion of LH and to a lesser extent FSH, results in testosterone deprivation, which subsequently leads to impaired spermatogenesis, decreased testis size and affected male behavior. In females, the lack of gonadotropins causes reduced gonadal steroid levels, reduced follicular growth, inhibition of ovulation, and reduced size of ovaries and uterus.

In male pigs, GnRH immunization has succesfully been applied as an alternative for surgical castration to prevent the occurrence of boar taint in pork (Meloen et al., 1994; Bonneau and Enright, 1995). Boar taint is mainly caused by accumulation of two compounds in the fat tissue; androstenone, which is a gonadal steroid, and skatole, a product of intestinal degradation of the amino acid tryptophane. The presence of both compounds in adipose tissue depends on the sexual maturity of the boar.

In humans, GnRH immunization has been studied for treatment of diseases which are driven by gonadal steroids, such as prostate cancer (Talwar, 1997, Simms et al., 2000). Application of immunization against GnRH has been described in many other species (see Chapter 2).

2.4 Passive immunization against GnRH

Active immunization against GnRH requires at least several weeks before an effective GnRH antibody titer is generated. In contrast, passive immunization results in a GnRH immunization effect within 24 hours (Caraty et al., 1984). This technique has been very helpfull in studying the pituitary-gonadal axis and the role of the hormones involved. For practical application, however, this approach is not suitable as large amounts of antisera and frequent administration are required to reduce hormone levels (Caraty et al., 1984; Van der Lende et al., 1992; Parthasarathy et al., 2002).

2.5 GnRH targeted toxins

Another approach to manipulate the pituitary-gonadal axis is to destroy GnRH receptor bearing cells using GnRH coupled to cytotoxins. Once GnRH has bound to its receptor, the conjugate is internalized, where the toxin than can inhibit protein synthesis, eventually leading to cell death. GnRH conjugated to pokeweed antiviral protein (PAP) proved to be cytotoxic in prostate cancer cell lines (Qi et al., 2003). In dogs, a single treatment reduced testosterone levels to zero for approximately 20 weeks. Thereafter, testosterone returned to

normal levels, indicating reversibility (Sabeur et al., 2003). Furthermore, little toxicity to normal intact tissue has been reported in animal studies (Harrison et al., 2004). This approach has been approved by the US Food and Drug Administration for other target molecules involved in cancer treatment (Harrison et al., 2004).

3. This thesis

3.1 Aim of the thesis

Vaccination against the hormone GnRH blocks the hypothalamic-pituitary-gonadal axis. Therefore, it can be used as an alternative for castration and fertility control. This has been described for farm animals (Bonneau and Enright, 1995; Dowsett et al., 1996), companion animals (Ladd et al., 1994; Robbins et al., 2004) and wildlife species (Millar et al., 1997; Millar et al., 2000). Application of GnRH vaccination in humans has been described for controlling fertility-related endocrine disorders and gonadal steroid-dependent diseases (Gual et al., 1997; Talwar, 1997; Simms et al., 2000).

During the past 3 decades, several attempts have been made to develop a vaccine against GnRH. However, limitations of such vaccines with respect to efficacy, i.e. ability to raise an effective antibody response followed by a blockade of gonadal steroid secretion in each vaccinated individual, has hampered application of these vaccines.

A peptide with an improved immunogenicity has been designed, by enlarging the GnRH molecule to a tandem peptide with twice the amino acid sequence of endogenous GnRH. This GnRH-tandem peptide conjugated to the carrier protein Keyhole Limpet Hemocyanin (KLH) induced a complete deprivation of serum testosterone in all treated piglets, while native GnRH conjugated to KLH was only partially effective (Meloen et al., 1994). All piglets treated with the GnRH-tandem conjugate showed a major reduction in testis weight (Figure 2 and 3). However, less than full efficacy was obtained when the number of animals treated increased.

The aim of the studies described in this thesis was to develop a highly immunogenic GnRH antigen and to evaluate the effects of this antigen in the target species. Emphasis has been put on (i) the development of an improved GnRH vaccine based on the previously developed GnRH-tandem peptide by dimerization of the antigen, introduction of amino acid substitutions and evaluation of the specificity of the induced antibodies, (ii) effects of GnRH vaccination on growth performance in pigs, (iii) application of the vaccine in horses and (iv) efficacy and safety of the vaccine proposed for clinical trials assessed in male pigs.

Figure 2. GnRH-immunized (right) and intact male pig (left)

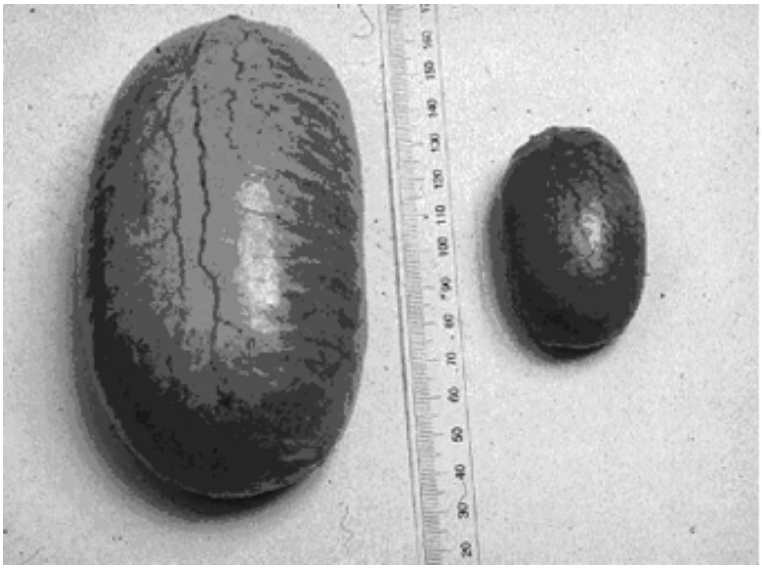

Figure 3. Testis of GnRH-immunized (right) and intact male pig (left).

3.2 Outline of the thesis

In Chapter 2, the literature on GnRH immunization has been reviewed.
In Chapter 3, effects of dimerization of GnRH (-tandem) peptides and the introduction of foreign amino acids on immunogenicity of the peptides was studied. Furthermore, effects of conjugation of the optimized antigen (G6k-GnRH-tandem-dimer) to a carrier protein were studied.
In Chapter 4, the role of the individual amino acid residues in the G6k-GnRH-tandem-dimer was investigated by replacing each amino acid one at a time by alanine and testing of the ability of these constructs to block the pituitary-gonadal axis in male piglets.
Recently, additional isoforms of GnRH were identified in mammals. The functions of these isoforms are still ill defined. In order to circumvent possible undesired effects of GnRH immunization on the unknown functions of these isoforms, cross-reaction of the induced GnRH-antibodies with the GnRH-isoforms must be prevented. In chapter 5, the specificity of antibodies raised against a panel of GnRH antigens for the different GnRH-isoforms was studied.
In male pigs, immunization against GnRH to prevent the occurance of boar taint at slaughter, is preferably performed around the end of the fattening period, in order to utilize maximally the boar-type growth during the period before the onset of effective immunization. However, immunization shortly before slaughter does not allow discrimination between effectively immunized boars and intact boars by simple visible inspection of the testis size, which we consider as a requirement for practical application of GnRH vaccination in pigs. In Chapter 6, therefore vaccine efficacy and growth performance in pigs that were effectively immunized at two different time points during the fattening period, was studied.
Since in horses no appropriate non-invasive methods are available to block the fertility axis, GnRH vaccination could be a suitable methode. In Chapter 7, two new immunization protocols employing the G6k-GnRH-tandem-dimer-OVA conjugate in aqueous adjuvant formulations were studied in sexually mature pony stallions. Efficacy was determined by GnRH specific antibody responses, hormone levels and effects on spermatogenesis and testis function.
In order to apply the GnRH vaccine for treatment of prostate cancer in men, the vaccine has been modified with respect to carrier protein. In chapter 8, efficacy and safety of the modified vaccine was studied. Different antigen doses administered in two adjuvants were evaluated at three different timepoints after vaccination in male pigs for their ability to establish neutralization of the pituitary-gonadal axis. Possible side effects of the vaccinations were determined by clinical observations, hematology, clinical biochemistry, urinalysis and macroscopic examining of organs and tissues.

In chapter 9, the results described in this thesis are summarized and the perspectives are discussed.

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Chapter 2

Active immunization against gonadotropin-releasing hormone (GnRH) in mammals: a review

ABSTRACT

Active immunization against gonadotropin-releasing hormone (GnRH) has been studied as a tool to block gonadal steroid production in mammals for more than 3 decades. This paper reviews the different aspects of vaccination against GnRH, i.e. vaccine development, biological aspects and application in veterinary and human medicine. Vaccine design has evolved from the use of endogenous GnRH protein conjugates emulsified with CFA to (genetically) modified peptide constructs in novel adjuvant formulations. Potentially, these vaccines can be employed as antifertility agents in veterinary practice, animal production and wild life control. Moreover, several vaccines are in development for treatment of prostate cancer in humans. However, sofar, lack of efficacy has hampered commercialization of these vaccines, presumably due to the use of weak adjuvants, low antigen doses or low immunogenicity of the antigen.

ABBREVIATIONS

Alum - Aluminum Hydroxide
BSA - Bovine Serum Albumin
CFA - Complete Freunds adjuvant
DDA - Dimethyldioctadecylammonium Bromide
DEAE Dextran - Diethylaminoethyl Dextran
DT - Diphteria Toxoid
FDA - Food and Drug Administration (US)
FSH - Follicle Stimulating Hormone
GnRH (LHRH) - Gonadotropin Releasing Hormone
HSA - Human Serum Albumin
IFA - Incomplete Freunds Adjuvant
ISA - Incomplete Seppic Adjuvant
KLH - Keyhole Limpet Hemocyanin
LH- Luteinizing Hormone
LPS - Lipopolysaccharide
MDP - Muramyl Dipeptide
MPL - Monophosphoryl Lipid A
OVA - Ovalbumin
PSA - Prostate Specific Antigen
TT - Tetanus Toxoid
SPF – Specific Pathogen Free

1. VACCINE DESIGN

Succesfull vaccination against an endogenous molecule requires a sufficient level of neutralizing antibodies during the full treatment period, to obtain the desired effect. GnRH, an endogenous molecule, is a small peptide, which consists of only 10 amino acids. Raising an immune response against such a small endogenous peptide, which is not immunogenic by itself, requires more than a standard approach. This means that coupling of GnRH to a carrier protein and the use of a strong adjuvant are not sufficient. Due to these limitations, the efficacy of the vaccine is likely to be insufficient to obtain a full response, i.e. complete cessation of gonadal steroid secretion in all individuals treated. Thus, the immunogenicity of the antigen must be amplyfied in order to develop an effective vaccine. The more so because, the use of strong adjuvants often results in injection-site reactions, which are undesired or even unacceptable in case of animals used for meat production.

1.1 Adjuvant

The adjuvants most commonly used in human and veterinary vaccines are oil-based adjuvants and aluminum hydroxide (Alum). Responses to Alum are often low and of short duration. Oil-based adjuvants are effective in generating a high immune response, but may cause inflammatory reactions. Oil-based vaccines mainly consist of an emulsion of oil, either mineral oil or non-mineral oil, and a waterphase, which contains the antigen. In general, water-in-oil emulsions give a higher immune response than oil-in-water emulsions. Complete Freunds adjuvant (CFA) is a mineral oil, which forms a water-in-oil emulsion, and contains killed and dried bacteria to stimulate the immune response. This combination induces high antibody responses; because of these characteristics and CFA being one of the oldest adjuvants used, it is ´the golden standard´ among adjuvants. However, due to the inflammatory side effects, which may occur at the site of injection, its use is mostly restricted to immunization studies in laboratory animals.
Instead of whole bacteria, bacterial compounds such as muramyl dipeptide (MDP), lipopolysaccharide (LPS) or monophosphoryl lipid A (MPL) can be used to stimulate the immune system. Alternative immune stimulating compounds are saponins, i.e. Quil A and the purified QS21 fraction, bacterial DNA, microparticles, Iscoms, liposomes, virus-like particles, block polymers and dimethyldioctadecylammonium bromide(DDA) (Singh and O´Hagan, 1999).
In several GnRH immunization studies, alternatives for CFA have been evaluated. In rats, Alum appeared to be only slightly less effective as compared to CFA (Ferro et al., 1996), however, in cattle CFA was superior to Alum (Goubau et al., 1989a). In cattle, the use of CFA induced a high antibody response but also caused granulomas at the site of injection, whereas DEAE Dextran (glucose polymer derivative) in combination with a mineral oil resulted in similar antibody titers as CFA with less side effects (Duggan et al., 1992). In a

comparitive study in mice, rabbits and goats, CFA was compared with Titermax, which contains non-mineral oil and a block polymer and forms a water-in-oil emulsion, and RIBI adjuvant, which contains non-mineral oil with microbial components and forms an oil-in-water emulsion (Bennett et al., 1992). Titermax induced antibody titers against GnRH equivalent to CFA in all three species investigated, while titers induced by RIBI were substantially lower. Inflammatory reactions induced by Titermax were mild and transient compared to those induced by CFA. Kiyma et al. (2000) compared CFA and ISA 51, which forms a water-in-mineral oil emulsion, and found that CFA was superior to ISA 51 with respect to antibody titers and subsequent effects on testosterone levels when tested in sheep. In contrast, others found effective GnRH antibody responses using ISA 51 combined with DDA in baboons (Finstad et al., 2004). In conclusion, effective antibody titers can be generated with adjuvants other than CFA, however, responses may differ among studies due to differences in target species, number of immunizations, antigen type and dose.

1.2 Antigen

Several approaches have been made to increase the immunogenicity of the antigen in order to be able to use a permitable adjuvant without making concessions to vaccine efficacy.

1.2.1 Carrier proteins

The most often used approach to make a peptide immunogenic, is to couple it to a protein molecule. Commonly used carrier proteins are KLH, TT, DT, OVA, BSA and HSA. The origin of the carrier protein could be of importance for the level of immunogenicity of the conjugate. The use of 'foreign' proteins is expected to result in conjugates with a stronger immune response. For instance, Hoskinson et al. (1990) showed that OVA was superior to BSA and HSA in bull calves and Ferro and Stimson (1998) found a higher antibody response with TT compared to thyroglobulin in rats. On the other hand, Goubau et al. (1989b) showed that conjugates prepared with the non-mammalian proteins KLH and TT, were less effective in raising high GnRH antibody titers in sheep than conjugates containing equine serum albumin and OVA. In general, most exogenous proteins can be used as carriers, although non-mammalian proteins are expected to be more immunogenic. The chemical linking of the peptide to the carrier protein is mainly done through a carbodiimide coupling (Jeffcoat et al., 1982), which links the peptide to the protein predominantly by carboxyl- and amino groups. Other conjugation reactions are the maleimidosuccinimide coupling which requires an additional cysteine or a sulfhydryl group in the peptide for coupling to an amino group of the protein (Meloen et al., 1994) or the glutaraldehyde coupling which requires an amino- or sulfhydryl group in the peptide (Talwar, 1997).

The site of conjugation may determine the efficacy of the immunization. Ladd et al. (1990) studied conjugation of GnRH to TT via glutamine at position 1, a substituted D-lysine at position 6 or Gly-OH at position 10. Conjugation via glutamine at position 1 induced a higher GnRH specific antibody response and reduced testosterone levels in rabbits more effectively than conjugation via positions 6 or 10. Substitution of the glycine at position 6 by a D-lysine for conjugation to DT has proven to be an suitable immunogen in several species, including humans (Giri et al., 1991; Gual et al., 1997; Talwar, 1997). Goubau et al. (1989b) observed no difference in GnRH antibody response in male sheep, when GnRH was conjugated to KLH via a substituted cysteine either at position 1, 6 or 10. Silversides et al. (1988) confirmed these results in mice. Although the GnRH antibody titers were similar for the 3 conjugates, specificity of the antisera depended on the site of conjugation. Conjugation via cysteine on position 1 resulted in C-terminal directed antibodies, conjugation via cysteine on position 10 generated N-terminal directed antibodies, while conjugation via cysteine at position 6 generated both N- and C-terminal antibodies. In contrast, Ferro et al. (2002a) showed that N-terminal conjugation via an cysteine substitution at position 1 resulted in effective immunization of rats, while conjugation via cysteine substitution at position 10 was not effective. Other groups used native GnRH extended with glycine and cysteine, conjugated to a carrier protein (Finnerty et al., 1994; Enright and Swift, 1995; Ferro et al., 1995; Miller et al., 2000) or longer spacer peptides (Simms et al., 2000; Parkinson et al., 2004). Thus, it seems that immunization with GnRH peptides, conjugated to a carrier protein via the N-terminus results in more effective antibody titers than conjugation via the C-terminus. However, this was not confirmed in all studies and may depend on the chemical approach used and substitution of amino acids required for coupling.

1.2.2. GnRH fusion proteins

To obtain a more defined antigen with a homogenous structure, some research groups focussed on recombinant proteins with multiple GnRH inserts. Van der Zee et al. (1995) vaccinated female rats and bull calves with purified hybrid GnRH fimbriae from E. Coli. This antigen generated an effective antibody response after two immunizations when administered in an oil-in-water emulsion. A fusion protein containing a domain of Pseudomonas exotoxin A and 12 copies of GnRH, generated high antibody titers in female rabbits and caused atrophy of the ovaries (Hsu et al., 2000). Quesnell et al. (2000) studied 2 fusion proteins based on ovalbumin and thioredoxin. In mice, both constructs were partially effective, when given seperately, while higher responses were observed in animals which received a combination of both proteins. Zhang et al. (1999) showed that immunogenicity increased with the number of GnRH inserts in the ovalbumin fusion protein. Proteins with 7 GnRH inserts performed better than proteins with 4 inserts. A GnRH fusion protein based

on leukotoxin administered in a commercially available adjuvant, reduced serum testosterone concentrations to undetectable levels in pigs (Manns and Robbins, 1997) and in cats (Robbins et al., 2004). In cattle, 3 vaccinations were required to reduce testosterone concentrations (Cook et al., 2000). In conclusion, efficacy of GnRH fusion proteins varies, which may be due to the immunogenicity of the protein or the number of the GnRH inserts in the protein.

1.2.3. Polyvalent GnRH peptides

Repetition of B-cell epitopes may enhance the immunogenicity of the antigen. Therefore, a GnRH-tandem peptide was designed, containing twice the amino acid sequence of endogenous GnRH (Meloen et al., 1994). This GnRH-tandem peptide conjugated to KLH induces a high GnRH neutralizing antibody response, subsequently leading to undetectable testosterone levels in all treated animals, whereas native GnRH conjugated to KLH was only partially effective. Others also observed enhanced immunogenicity of dimerized GnRH peptides (Ferro and Stimson, 1998), although the efficacy of the immunization depended on the site of dimerization. Dimerization via a C-terminal cysteine resulted in a dimer which was more immunogenic than its monomeric equivalent when conjugated to a carrier protein, whereas dimerization via the N-terminal cysteine resulted in a less immunogenic molecule as compared to its monomeric equivalent (Ferro et al., 2002b). Polyvalent constructs can also be generated using the so-called multiple antigenic peptide (MAP) (Tam, 1988). GnRH-tandem peptides coupled to these lysine-branched constructs and emulsified in CFA, were effective in reducing testis size in male pigs (Beekman et al., 1999).

1.2.4 Synthetic GnRH-T cell epitope constructs and lipopeptides

Completely synthetic peptide antigens containing a T cell epitope and the GnRH decapeptide were tested in several studies, mainly in mice. Mice receiving the GnRH peptide extended with a T cell epitope from the influenza virus hemegglutinin at the N-terminus were infertile for more than 24 weeks after two vaccinations using CFA as adjuvant (Ghosh and Jackson, 1999). Zeng et al. (2001a) studied the effects of the ligation chemistry used for preparation of the synthetic peptide construct comprising a T cell epitope and GnRH. Lineair synthesis, thioether bond formation and oxime bond formation resulted in more immunogenic peptide constructs than peptides linked via a disulfide bond. In a subsequent study (Zeng et al., 2002a), lipids were attached to the GnRH-T cell epitope constructs in order to develop lipopeptides which could be used without an additional adjuvant. The lipids developed in this way were a synthetic version of the N-terminal part of a lipoprotein from gram nagative bacteria, dipalmitoyl-S-glyceryl-cysteine (Pam2Cys) and tripalmitoyl-S-glyceryl-cysteine (Pam3Cys). Some of the tested lipopeptides were

highly immunogenic and resulted in efficient sterilization of female mice when administered in saline either via the subcutaneous or intranasal route. In our experiments, a conjugate comprising a GnRH-tandem peptide and Pam3Cys dissolved in PBS was not able to block testis growth in any of the treated male pigs (Turkstra et al., unpublished results). Beekman et al. (1999) studied the effects of the nature of the bond between peptide and lipid on the immunogenicity of these constructs. Coupling of a GnRH-tandem peptide to palmitic acid via an a thio-ester bond appeared to be more effective than coupling via an amide bond. The former construct administered in CFA inhibited testis growth in 17 out of 23 treated pigs, while the latter was only effective in 8 out of 20 pigs. In conclusion, GnRH-T cell epitope constructs render mice infertile when administered in CFA, while attachment of lipids, resulted in an immunogenic compound which was effective without an additional adjuvant in mice. In pigs, GnRH lipopeptides require the help of an adjuvant in order to block testis development.

2. BIOLOGICAL EFFECTS OF GnRH IMMUNIZATION

GnRH immunization has been used to study the effects of hormones involved in the regulation of the hypothalamic-pituitary-gonadal axis. In particular the absence of GnRH at the pituitary site has provided valuable information (see below). Challenge of GnRH immunized animals with GnRH analogs, gonadotropins or gonadal steroids revealed the role of these hormones in pituitary-gonadal function (see 2.3 – 2.5). Effects of GnRH immunization on pregnancy and reversibility of the effects induced by GnRH immunization are summarized in section 2.6 and 2.7, respectively.

2.1 Receptor concentrations and hormone content in GnRH immunized mammals

The role of GnRH in the long-term control of GnRH receptor concentrations was studied by immunoneutralization of GnRH. In a study in female rats, GnRH immunization reduced the number of GnRH receptors in the pituitary (Popkin and Fraser, 1985). A reduction in pituitary GnRH receptor concentration and pituitary GnRH receptor mRNA was also found after passive immunization of castrated male sheep. This indicates that GnRH stimulation is required to maintain GnRH receptor concentrations (Sakuria et al., 1997). In rhesus monkeys, GnRH immunization reduced the hypothalamic GnRH content (Chappel et al., 1980). In contrast, Rabb et al. (1990) observed no effects of GnRH immunization on GnRH content in the median eminence or the body of the hypothalamus in castrated male ponies. Adams and Adams (1986) used ovariectomised ewes to study the effect of GnRH immunization on pituitary LH and FSH content. The pituitary content of both gonadotropins was significantly reduced by GnRH immunization. Similar results were found in GnRH immunized castrated male ponies (Rabb et al., 1990) in which LH and FSH

content were reduced by 90% and 55%, respectively. Awoniyi et al. (1988a) showed that GnRH immunization of boars reduced pituitary LH content, but did not affect the FSH content. This is probably due to the fact that in this study, GnRH immunization did not affect FSH, as FSH levels in circulation were normal. In rhesus monkeys, GnRH immunization also depressed pituitary LH content (Chappel et al., 1980). FSH concent was not determined in this study.
Thus, GnRH immunization reduces the GnRH receptor concentration and the LH content in the pituitary, while the pituitary FSH content and hypothalamic GnRH content were either not affected or only partially influenced.

2.2 Castration of GnRH immunized mammals
Besides the biological effects of GnRH immunization on hormone levels and gonadal function, efficacy of vaccination can also be determined by surgical castration of immunized animals.
Castration of intact male and female mammals results in a so-called post castration rise in LH and FSH, due to the lack of feed back of gonadal steroids. As expected, surgical castration of mammals effectively immunized against GnRH does not induce such a response (Jeffcoat et al., 1982; Garza et al., 1988).

2.3 GnRH challenge in GnRH immunized mammals
Challenge of intact mammals with GnRH induces a LH and FSH response. In mammals effectively immunized against GnRH, high antibody levels prevent such a response (Garza et al., 1986). In animals that regain fertility after a period of effective immunization, the response appeared to be correlated with the recovery: ramlambs with testes weighing more than 100 gram showed higher LH and testosterone levels after a GnRH challenge than animals with testis weighing less than 70 gram (Brown et al., 1994).

2.4 GnRH agonist challenge in GnRH immunized mammals
Challenge of GnRH immunized animals with a GnRH agonist, which is not neutralized by the antibodies raised, causes activation of the GnRH receptor and subsequently secretion of gonadotropins. In effectively immunized animals responses to the challenge are impaired. In GnRH immunized sows for example, LH and FSH increased to levels which were approximately 25% of the levels seen in controls (Esbenshade and Britt, 1985). In castrated male ponies immunized against GnRH, the increase in LH was only 10% of the response observed in the controls after a GnRH challenge, while the FSH response was similar for immunized and control animals (Rabb et al., 1990). In contrast, challenge of passively immunized ovariectomized sheep with a GnRH agonist induced pretreatment like LH surges (Caraty et al., 1984). The impaired LH and FSH peak values in effectively

immunized animals may be due to a reduction in the pituitary gonadotropin content (Jeffcoat et al., 1982) or a reduced number of GnRH receptors in the pituitary (Esbenshade and Britt, 1985).
In female mammals immunized against GnRH, pulsatile administration of GnRH agonists have been used to restore gonadotropin levels, follicle growth and ovulation. In ovariectomized ewes immunized against GnRH, pulsatile administration of a GnRH analogue resulted in elevated LH levels within 48 hours and established normal LH levels within 6 days (Adams and Adams, 1986). Pulsatile administration of 100 ng GnRH agonist per hour for several days did induce follicle growth, but not ovulation. Ovulation occured when the agonist dose was increased to 800 ng per hour (Sakuria et al., 1992).

2.5 Challenge of GnRH immunized mammals with gonadotropins or steroids
GnRH immunization has been studied for the use as a male contraceptive. Therefore, the effects of testosterone supplementation in order to maintain libido after GnRH immunization were investigated. Awoniyi et al. (1993) described a model for male contraception by immunization against GnRH in combination with small testosterone implants in rats. GnRH immunization reduced LH and FSH to undetectable levels, whereas a low dose of testosterone maintained libido without restoring fertility. Similar results were found by Ladd et al. (1988) in rats and rabbits. Awoniyi et al. (1992) showed that high doses testosterone were capable of restoring spermatogenesis and fertility in adult azoospermic rats without influencing serum LH and FSH levels. When testosterone supplementation was started after a long periode of suppressed fertility due to GnRH immunization, effects on spermatogenesis were less pronounced, spermatogenesis could only be partially restored (Esbenshade and Johnson, 1987; McLachlan et al., 1994).
The role of FSH in the regulation of spermatogenesis in the absence of testosterone was also studied in GnRH immunized rats. A challenge with recombinant FSH partially restored spermatogenesis, though spermatid elongation was not restored and may require additional factors, most likely testosterone (McLachlan et al., 1995).
In females, effects of gonadotropin supplementation on follicle development and ovulation were studied in GnRH immunized sheep. Immunization reduced FSH levels by 75% and as a consequence a decrease in the number of large follicles occured. Subsequent treatment with FSH, LH and hCG caused an increase in the number of large follicles and induced ovulation, however, the ovulation rate was lower in immunized animals compared to the controls (Mariana et al., 1998).

2.6 Effects of GnRH immunization on pregnancy
The role of GnRH in early pregnancy in the rat was studied by passive immunization using GnRH polyclonal or monoclonal antibodies. Administration of antibodies on day 4 of

pregnancy caused a delay in implantation of the embryos by 5 days, but pregnancy was not terminated. This effect could be abolished by administration of GnRH or estradiol (Arimura et al., 1976). Administration of GnRH antibodies between days 7 and 10 of gestation resulted in fetal death, emphasizing the importance of GnRH for the maintance of pregnancy as indirect stimulator of progesterone production (Nishi et al., 1976; Gupta et al., 1985). Passive immunization of late pregnant rats at days 13 and 20 of gestation did not terminate pregnancy but resulted in reduced size of the sex organs in the pups at day 6 after birth (Bercu et al., 1978). Passive immunization of ewes, 7 weeks before partus impaired LH pulses in the fetuses, whereas FSH levels were either not or slightly decreased (Miller et al., 1998).

2.7 Reversibility of GnRH immunization effects

Suppression of the reproductive axis by GnRH neutralization is due to a sufficient amount of neutralizing antibodies. Hormone levels and gonadal activity are regained when antibody titers drop below a certain level, which is insufficient to neutralize GnRH completely. Several examples demonstrating reversibility of GnRH immunization effects have been published. Complete reversibility of the effects induced by GnRH immunization were shown in male rats. Fertility returned to normal again between 6 to 10 months after the last vaccination, as determined by the production of litters with a normal size (Rovan et al., 1992, Ferro et al., 1995, Kumar et al., 2000). In horses, mares with suppressed ovarian activity for up to 30 weeks after GnRH immunization, conceived and produced normal foals in the following breeding seasons when antibody titers had decreased significantly (Tsewang et al., 1997), while in stallions recovery of testis function was observed and libido returned to normal again when GnRH immunization was discontinued (Dowsett et al., 1996, Van der Meer, 2000). In bull calves, testosterone and testes size increased to normal levels after 40 weeks of suppression (Robertson et al., 1981), while others observed an increase in aggressive and sexual behavior to bull-like levels when immunizations were stopped (Jago et al., 1997). In adult dogs with reduced serum testosterone levels, antibody titers declined to pre-immune levels after a period of 28 weeks following immunization and resulted in testosterone levels which restored to the normal range (Ladd et al., 1994).
In pre- and peripubertal immunized sheep, gonadal development was suppressed for more than 90 weeks due to low gonadotropin concentrations. Surprisingly, at that time antibodies could not be detected anymore, suggesting that other factors appeared to be involved in the sustained suppression of gonadotropine levels after GnRH immunization (Brown et al., 1994; Brown et al., 1995). In these animals, secretion of GnRH in portal blood, in particularly the GnRH pulse amplitude was reduced, but GnRH concentrations in the median eminence were similar for immunized and control animals at the time they had reached the age of 3-4 year (Clarke et al., 1998). The authors suggested that this

discrepancy could be caused by thickening of the bloodvessel walls in the median eminence or by immune cells 'attacking' the terminal buttons of the GnRH neurons, as was previously suggested by Molenaar et al. (1993). In this study in pigs, changes in the median eminence of GnRH immunized pigs were described. Damage was expressed in a lesions score, which was based on: i) a decrease in GnRH reactivity in the median eminence, ii) tissue disruption and fibrosis iii) increased accumulation of neurosecretum (Herring bodies), and iv) size of affected area of the median eminence.
Although the authors interpret the observed findings as lesions, it is not unlikely that the morphological changes can be attributed to a functional atrophy. This will result in a decreased number of GnRH positive cells in the median eminence and consequently, anatomical structures involved in secretion and transport of GnRH as neurons and capillaries are atrophic. The observed presence of an increased IgG immunoreactivity in the median eminence after vaccination, which also occurred in controle animals, has not lead to influx of inflammatory cells, a hallmark of inflammation and no pathological attributes of an autoimmune process have been shown.
Moreover, in a follow-up study with SPF-pigs, no evident autoimmune or inflammatory changes in the median eminance of GnRH immunized pigs were noted as compared to mock vaccinated control animals (Stockhofe et al., unpublished data). In a seperate experiment, histopathological examination of the hypothalamus of pigs effectively immunized against GnRH for 8, 16 or 26 weeks (see Chapter 8), revealed no pathological changes (unpublished results).
In general, reversibility of GnRH immunization effects is initiated once the antibody titers are below a certain threshold level. However, in young animals sustained effects may occur, which are possibly due to impairment of hypothalamic function.

3. APPLICATION OF GnRH VACCINES

In farm animals, vaccination against GnRH could be an alternative for conventional castration methods or to prevent sexual behavior in animals, which are normally not castrated. In pig husbandry, male piglets are surgically castrated to prevent the occurance of boar taint in pork. An alternative for the surgical castration of young boars is urgently needed, as castration is performed without anaesthesia, which makes it a painfull and animal unfriendly method. In horses, suppression of fertility and sexual behavior can be achieved by surgical castration or hormone administration. As both approaches have drawbacks, such as the risk of surgery or daily administration of hormones and their unknown long-term effects, a suitable alternative is desired. Fattening of bulls and heifers suffers from problems related to puberty: bulls show aggressive and sexual behavior, while heifers manifest oestrus behavior, resulting in stress and unwanted pregnancies. Separation

of males and females, surgical castration and hormone administration have been applied to overcome these problems. In male sheep and goats, several castration methods are used to prevent a reduction in growth rate, caused by sexual and aggressive behavior. In pets and wildlife species a GnRH vaccine could serve as a contraceptive, to prevent overpopulation and unwanted pregnancies and sexual behavior. In human health care, vaccination against GnRH could be a potential treatment for several gonadal steroid-dependent diseases, which are currently treated with GnRH agonists. It is expected that GnRH immunization has less side effects and requires lower doses.

3.1 Pigs

Surgical castration of young male pigs is common practice in pig farming. Castration is performed to prevent the occurrence of boar taint in pork (see 3.1.1). A considerable amount of research has been carried out in order to solve the boar taint problem (see 3.1.2), nevertheless the mutilating practice of surgical castration without anaesthesia is still allowed. A non-invasive alternative such as vaccination against GnRH is a suitable option (see 3.1.3), as was demonstrated by the approval of such a vaccine in Australia (Improvac, CSL Animal Health, Victoria, Australia, recently acquired by Pfizer Animal Health).

3.1.1 Boar taint

Boar taint is an unpleasant odour, which can be perceived when meat of male pigs is heated. The odour is mainly caused by two compounds in the fat tissue, androstenone and skatol (Patterson, 1968, Vold, 1970, Matthews et al., 2000). Male pigs reach puberty between 18-21 weeks of age (FlorCruz and Lapwood, 1978). Pubertal development is reflected by elevated steroid production by the Leydig cells in the testis, leading to increased testicular steroid levels in the blood stream, including the pheromone androstenone (Claus et al., 1994). Androstenone is mainly stored in the salivary glands and released in the saliva to induce receptivity of female pigs in estrous. Due to its lipophilic character, androstenone has a tendency to accumulate in the adipose tissue in much higher concentrations than the testicular sex hormones testosterone and estradiol (Claus et al., 1994).

Skatol is a product of protein processing in the gut of the pig, in particular the processing of the amino acid tryptophan. Skatole, absorbed from the gut, is metabolized by the liver and partly accumulated in fat tissue. Its presence in fat tissue depends on the sexual maturity of the male pigs (Bonneau et al., 1992), as its metabolism is affected by estrogens. Increased estrogen levels have an inhibitory effect on the metabolism of skatole by the liver and clearance from the body, resulting in increased levels of skatole in fat tissue (Babol et al., 1997). The involvement of estrogens in skatole formation in fat tissue also explains the higher skatole levels in boars compared to sows, as estrogen levels in boars are higher than

in sows (Claus and Weiler, 1994). Low voluntary feed intake due to high estrogen levels in boars, may also affect skatol concentrations as it lowers the intestinal passage, resulting in an increase in skatole formation (Claus et al., 1994). Skatole levels in adipose tissue are also influenced by other factors, such as the energy level of the diet (Claus et al., 1994), diet composition (Claus and Raab, 1999; Knarreborg et al., 2002; Claus et al., 2003), feed intake prior to slaughter (Andersson et al., 1995) and housing conditions (Hansen et al., 1995).
Both boar taint substances are perceived differently, the flavour of skatole is mostly related to 'manure' while androstenone is mostly perceived as 'sweat' or 'urine' (Dijksterhuis et al., 2000). For both substances androstenone and skatole threshold levels are assessed, 0.5 µg and 0.2 µg per gram fat tissue, respectively. Values above these levels are indicative for boar taint (Bonneau et al., 1992).

3.1.2 Alternatives for surgical castration of piglets

The occurance of boar taint makes the meat unsuitable for consumption, especially in case the meat has to be heated before consumption. The meat is also unfit for export, as most countries demand boar taint free meat. Surgical castration eliminates boar taint, but has consequences for the animal and the farmer. It is performed without anaesthesia and thus is an animal unfriendly practice which causes pain and has an impact on animal welfare (McGlone et al 1993, Von Waldemann et al., 1994). In addition there is increased susceptibility to diseases (De Kruijff and Welling, 1988) and it is economically undesirable, as it leads to a more inefficient feed conversion and a reduction in carcass quality (Walstra and Vermeer, 1993).
Alternative procedures should preferably improve animal welfare without further costs for the farmer. Several alternatives have been mentioned, although most have disadvantages. Surgical castration with local anaesthesia is an alterative, which reduces stress and discomfort of the piglet. No difference in behavior was observed in piglets surgically castrated with local anaesthesia as compared to intact piglets, whereas piglets castrated without anaesthesia exhibited altered behavior, indicative of pain and discomfort (Wemelsfelder and Van Putten, 1985, McGlone and Hellman, 1988, Mc Glone et al 1993). Moreover, stress caused by castration, as evaluated by a higher heart rate and vocalization, is reduced by local anaesthetics (White et al., 1995). However, surgical castration with the use of anesthesia requires veterinary assistance, which makes this approach costly and practically impossible.
Detection of boar taint at slaughter would be preferable as it allows the production of intact male pigs. In Denmark, slaughterline detection of boar taint was applied in the nineties, but only skatole levels were determined, which appeared to be insufficient to detect all tainted carcasses. Successful detection requires analysis of both substances, androstenone and

skatole. However, the lack of a high throughput system for measuring androstenone hampers application of this methode. The use of an electronic nose, which measures both substances at one occassion, has been successfully applied in an experimental setting (Annor-Frempong et al., 1998). All tainted carcasses were identified, however 16% of the carcasses were classified false positive. Although this alternative may look promising, it does not solve the problem of the high incidence of boar taint, which is about 60% in Europe (Walstra et al., 1999).
Slaughtering of pigs at a lower body weight certainly will reduce the incidence of tainted carcasses, but additional slaughterline detection is still necessary to identify tainted carcasses. At a body weight of 80-90 kg still more than 5% of the carcasses exhibits boar taint (Bonneau et al., 1987, Le Denmat et al., 1993). Moreover, lighter carcasses are less profitable for pig farmers and may affect pork export. Another possibility to control the occurence of boar taint is by selection for low concentrations of boar taint compounds. The use of sires and dams selected for low fat androstenone concentrations, lowered androstenone concentrations in the offspring as compared to pigs selected for high androstenone levels (Willeke et al., 1987). However, no significant differences with the control group were seen. Moreover, selection for low androstenone concentrations negatively affected growth rate, which can be explained by the fact that selection against androstenone is associated with late maturity.
Skatole levels in intact male pigs can be influenced by selection for growth rate on diets with different protein levels. Pigs selected for growth rate on a high protein diet had significantly lower skatole levels than pigs selected for growth rate on a low protein diet (Lundstrom et al., 1994). Although, the different protein sources used for both diets may also contribute to the observed differences in skatole levels.
Sperm sexing has proven to be effective in producing litters of the desired sex (Johnson, 1991). However, it is unlikely that this technique will be implemented in pig husbandry in order to produce predominantly female piglets. Conventional artificial insemination requires about 2 to 3 billion spermatozoa, which is far above the capability of sorted sperm production (Johnson et al., 2005).

3.1.3 Immunization against GnRH

To circumvent the disadvantages of surgical castration, immunization against GnRH seems a feasible option. Disruption of the hypothalamic-pituitary-gonadal axis by neutralization of GnRH, leads to a reduction of LH and FSH production and secretion, thereby inhibiting maturation of the testes in growing animals as well as the synthesis of testicular steroids among which androstenone, testosterone and estrogens. This approach makes surgical castration redundant as concentrations of the boar taint causing compounds, androstenone and skatole, are reduced to levels similar to surgical castrated pigs (Hennessy et al., 1997).

In boars, several studies have been performed, focussing on the occurance of boar taint in GnRH immunized boars. Falvo et al. (1986) used a vaccine consisting of GnRH conjugated to HSA with CFA or MDP as the adjuvant. Immunization at 12, 16 and 18 weeks of age resulted in undetectable LH and testosteron levels and reduced weight of testes and accessory sex glands. Moreover, the incidence of boar taint was reduced. Furthermore, pituitary and Leydig cell function were studied in immunized boars (Awoniyi et al., 1988a). Undetectable serum LH and testosterone levels were accompagnied by a reduction in pituitary LH content and testicular LH receptor content. However, plasma FSH concentrations and pituitary FSH content were not affected. In a subsequent study, the morphology of the testes of GnRH immunized boars was examined (Awoniyi et al., 1988b). Changes were seen in the seminiferous tubules: diameter and epithelium height were reduced, spermatids were absent and Sertoli cells structure was affected. Due to the depletion of LH, size, number and activity of the Leydig cells was reduced. Effects on sperm counts were studied in 11-month-old boars by Grizzle et al. (1987). Five immunizations with a GnRH-BSA conjugate in CFA reduced LH, testosterone and testes volume. In these mature boars, testes weight was reduced by 66% and the number of sperm cells was reduced by more than 75%.

In a study by Bonneau et al. (1994), growth performance of GnRH immunized boars was studied. Immunization at 29 and 89 kg body weight with a GnRH conjugate in mineral oil and in an aqueous solution with saponin as an adjuvant, respectively, resulted in highly variable antibody titers, ranging from undetectable to substantial levels. Mean testis weight was reduced as were plasma testosterone concentrations and adipose androstenone levels, while growth performance, i.e. average daily weight gain and feed efficiency, of the immunized boars was similar to that of intact boars and improved compared to surgical castrates.

Our group demonstrated that testis size or testis weight could serve as a parameter for absence of boar taint in immunized pigs (Oonk et al., 1995). Pigs immunized against GnRH at 10 and 18 weeks of age and slaughtered at 26 weeks of age, always had androstenone levels below 0.5 μg/g when testis weight was less than 150 g or testis size measured prior to slaughter was less than 9 cm.

In Chinese cross-bred pigs, a modified GnRH-tandem-dimer molecule conjugated to OVA (as described in Chapter 3 of this thesis) and emulsified in Specol adjuvant (Bokhout et al., 1981), decreased serum testosterone and adipose androstenone levels, and caused a reduction in testis weight in 35 out of 39 treated male pigs (Zeng et al., 2001b, 2002b). Effects of this vaccine on growth performance were studied in pigs fed diets with a high or a low energy content. For both diets, feed intake of the immunized boars was similar to the surgically castrated littermates, while the energy conversion ratio was in between those of

intact boars and surgical castrates. This resulted in a growth rate of the immunized boars which tended to be higher than intact boars and surgical castrates (Zeng et al., 2002c).
A recombinant fusion protein containing leukotoxin and several copies of a GnRH-tandem molecule in a commercially acceptable oil-in-water adjuvant was also evaluated as a boar taint vaccine (Manns and Robbins, 1997). In this study, boars received the first vaccination at 3 weeks of age and a booster when they reached 100 kg bodyweight. This vaccination lowered serum testosterone and fat androstenone concentrations to castrate levels in all 11 treated pigs. However, no follow-up studies in pigs have been published using this vaccine.
The first commercially available boar taint vaccine, Improvac (CSL Animal Health, Victoria, Australia), was approved in 1998 for prevention of boar taint in male pigs in Australia. Castration is not common in Australian, however, the export of pork to Japan required the production of heavier animals. In order to avoid a high incidence of boar taint carcasses, a vaccine against GnRH was introduced. The vaccine comprises a GnRH peptide conjugated to a carrier protein, mixed with non-oil based adjuvant. More details about the vaccine were not published. Two doses of 2 ml vaccine are advised to be administered 8 and 4 weeks before slaughter to efficiently reduce boar taint. The vaccine should be administered using special equipement, the Improvac vaccinator gun, thereby injecting the vaccine in the skin.
Dunshea et al. (2001) studied the effects of Improvac in boars slaughtered at 23 or 26 weeks of age. Testosterone levels were reduced after the second vaccination, however, at time of slaughter still 8% of the immunized boars had testosterone levels above 2 ng/ml, which is substantially higher than the levels in castrated pigs (0.3 ng/ml). Mean testis weight of Improvac-treated boars was reduced by 50% as compared to intact controls. The authors stated that in pigs treated with Improvac, testes weight should be less than 350 and 400 gram for pigs slaughtered at 23 and 26 weeks of age, respectively, to ensure boar taint free carcasses. However, based on these threshold levels it is not possible to discriminate between treated and untreated boars, as a substantial number of the untreated boars had testes weights below these values. Growth performance of the Improvac-treated boars was altered after the second vaccination. Treated boars grew faster than intact boars and had better feed conversion efficiency and leaner carcasses than castrates. Moreover, Improvac-treated boars were less aggressive, only 4% exhibited wounds around the head and shoulders due to fighting compared to 26% of the intact boars. Tissue reactions due to injection of the vaccine were observed in 15 to 20% of injection sites. Androstenone and skatole levels of Improvac treated boars were significantly reduced as compared to intact boars and were similar to castrated boars. However, 6% of the Improvac-treated boars that were slaughtered at 26 weeks of age had androstenone levels exceeding the threshold of 0.5 µg/g backfat and 2% had skatole levels above 0.2 µg/g backfat. In a field study including

319 vaccinated boars, 2.5% and 2% of the vaccinated pigs had values above the treshold for androstenone and skatole, respectively (Hennessy et al., 1997).

3.2 Horses

3.2.1 Alternative for castration

In horses, GnRH immunization can be used as an alternative for surgical castration of stallions. Surgical castration, although commonly applied, is not without risks as it causes complications in more than 5% of the treated stallions (Keller and Hartmann, 1996). Castration of cryptorchid stallions even has a higher risk for complications (Searle et al., 1999). Therefore, a non-surgical castration method without the risk for complications is preferable. Several hormone treatments have been applied in order to reduce libido and aggresive behavior, but efficacy of these treatments is limited, costs are high and frequent administration is required (Roberts and Beaver, 1987, Hinojosa et al., 2001).

An advantage of GnRH immunization is the reversibility of the induced effects. When GnRH immunization is not repeated, a recovery of the testis function can be observed and libido will return to normal again (Dowsett et al. 1996). Such a reversible ´castration´ method can save the reproductive capacity of exclusive stallions that are involved in all kinds of sports and normally will be surgically castrated because of their undesired libido and agressive behavior.

As in stallions, the most important reason for GnRH immunization studies in mares is prevention of undesirable sexual behavior. Behavioral changes may interfere with performance in sporting competition and with handling of the mares. In addition, some mares exhibit continues estrous behavior, due to hormonal changes.

GnRH immunization may be considered an alternative for castration for medical reasons, e.g. for treatment of persisting equine arteritis virus (EAV) infections in stallions. After the acute phase of the disease, infection of stallions frequently leads to a carrier state in which virus is continually shed with the semen. The presence of testosterone is essential for the persistence of the virus in the stallion (McCollum et al., 1994). A temporarely intervention in testosterone production, as established by GnRH immunization, could be sufficient to clear the infection. Infections in mares are usually mild and transient, but may lead to termination of pregnancy.

3.2.2 Immunization against GnRH

The first GnRH immunization study in horses was performed by Schanbacher and Pratt (1985), who succesfully treated a cryptorchid stallion; testosterone concentration was reduced and the stallion behaved docile. In this study, CFA was used as an adjuvant which is not allowed be used in practice, as it may induce severe reactions at the site of injection. Local reactions at the injection site were also observed in colts immunized with a mineral

oil-based GnRH-vaccine (Dowsett et al., 1991). In this study all treated stallions showed depressed testosterone levels and testis function appeared to be affected. However, the occurance of adverse reactions after vaccination is not acceptable for horse owners, therefore the vaccine should preferably contain an adjuvant which does not induce injection site reactions. Dowsett et al. (1996), tested a GnRH vaccine containing a water-soluble adjuvant in young stallions. Two vaccinations caused a decrease in testosterone concentrations and absence of libido, which lasted for 3 to 6 months. Unfortunately, this vaccine has not been applied in sexually mature stallions, which most likely will be the most important target group. Malmgren et al. (2001), treated three adult stallions with a GnRH vaccine comprising Equimune adjuvant. This vaccine failed to induce high antibody titers in one of the three stallions, despite the fact that five immunizations were given. Moreover, local tissue reactions were observed in all treated stallions.
In mares, immunization against GnRH suppressed ovarian activity (Garza et al., 1986; Safir et al., 1987). In these studies CFA was used as an adjuvant, whereas the use of Equimune adjuvant resulted in a partially effective vaccination (Dalin et al., 2002). A water-soluble vaccine was shown to suppress fertility for 20 weeks in 4 young mares (Dowsett et al., 1993). Recently, CSL Animal Health (Victoria, Australia) launched a GnRH vaccine, called Equity, for estrous control and prevention of estrous related behavior in mares. Vaccination results in suppression of ovarian activity subsequently leading to reduced estrous behavior, however, according to the medication guide results are variable: not all mares respond (www.pfizeranimalhealth.com.au/CSLAnimalHealth).

3.3 Cattle

Bulls grow more efficiently than castrated bulls and produce leaner carcasses. However, to prevent aggressive and sexual behavior, most bulls fattened in pasture based systems and feedlots are castrated. Several castration methods can be applied, which all cause stress and pain (Fisher et al., 1996). In beef cattle, pregnancy and estrous behavior impairs growth rate, whilst estrous and pregnancy in dairy cattle to be culled, cause a reduction in milk production. Immunization against GnRH has been studied in order to suppress fertility and sexual behavior in cattle. Robertson et al.(1979) showed that GnRH immunization reduced testosterone levels in bull calves. Further studies showed docile behavior of the treated bull calves, improvement of growth performance as compared to castrated bulls and reversibility with respect to hormone levels (Robertson et al., 1981, 1982). In beef heifers, GnRH immunization was effective in preventing pregnancy (Johnson et al., 1988). In these studies, multiple vaccinations were given with CFA as the adjuvant.
A single immunization protocol was tested by Adams and Adams (1992). Five mg GnRH-KLH conjugate in CFA reduced testes weight in 12-month-old bulls, however, at slaughter at 18 months of age, the vaccine appeared not to be effective anymore: testosterone levels

were again at the same level as those of the untreated bulls. Similar results were found when a single immunization was given at a younger age, i.e. at 4 or 7 months of age (Adams et al., 1996). Two immunizations, the first one at 1, 4 or 6 months of age and a booster at 12 months, did result in reduced testosterone levels at slaughter (Huxsoll et al., 1998). In these bulls, growth performance was in between that of intact and castrated bulls, while aggressive behavior was reduced to castrate-like levels (Price et al. 2003).
In beef heifers, Adams and Adams (1990) compared the use of GnRH immunization and daily oral hormone administration (melengestrol acetate, MGA) in order to prevent estrous behavior and pregnancy. Both, MGA treated heifers and heifers treated with the GnRH-KLH conjugate in CFA were not cyclic. Weight gain and feed efficiency were not different between intact, MGA treated and GnRH immunized heifers, indicating that GnRH immunization can be used as an alternative for MGA administration.
Vaxstrate, a GnRH vaccine, which consists of a GnRH decapeptide conjugated to OVA in a DEAE Dextran/mineral oil mixture, was approved in Australia to prevent estrous behavior and pregnancy in cattle (Hoskinson et al., 1990). Five ml emulsion must be administered subcutaneously, followed by a booster immunization 4 to 16 weeks later. Although pregnancy rates were low in case of a vaccination interval of 4 weeks, a 16 week vaccination interval resulted in pregnancy rates of 20%, while pregnancy rates in the control group were 63%. The effects of Vaxstrate on growth performance were studied by Jeffery et al. (1997). Growth performance and carcass value of Vaxstrate treated heifers appeared to be in between those of intact and surgically spayed heifers. However, the costs of spaying were less than the costs for GnRH vaccination, 4 vaccinations at 5.5 Australian dollars each, making GnRH immunization a less attractive alternative. Jago et al. (1997) immunized bulls with Vaxstrate at 2, 4 or 7.5 months of age and boostered 1 or 2 times several weeks later. Two or 3 immunizations appeared not to be sufficient to suppress testosterone levels and sexual behavior until slaughter at 18 months of age.
Prendiville et al. (1995) evaluated a single immunization with a Vaxstrate-like vaccine containing a GnRH-HSA conjugate in a DEAE Dextran/mineral oil mixture with bacteria incorporated. Immunization of 8-month-old heifers delayed puberty by 3.5 month, but also reduced growth performance. When the conjugate was administered in DEAE Dextran without mineral oil, 2 immunizations were required to reduce testosterone for a similar periode in bull calves (Finnerty et al., 1994). Though antibody responses were variable in these bulls, aggressive and sexual behavior was reduced (Finnerty et al., 1996).
A mixture of two GnRH fusion proteins, based on ovalbumin and thioredoxine, administered in CFA suppressed LH and testosterone concentrations in bulls after the second vaccination (Aissat et al., 2002), while in heifers, 3 vaccinations with the antigens in a water-in-oil emulsion containing bacteria as an immunostimulant, resulted in a ceasation of the estrous cycle in all 39 treated animals (Stevens et al., 2005) Immunization with a

recombinant fusion protein consisting of leukotoxin and several copies of GnRH was studied using a commercially acceptable adjuvant (Cook et al., 2000). Due to variable responses, mean testosterone levels were only slightly reduced. The authors concluded that more research is needed to reduce variability in the response to the vaccination and to optimize the timing of vaccination.

3.4 Sheep and goats

In two early GnRH immunization studies in ramlambs, a GnRH-BSA conjugate in CFA was used. Jeffcoat et al. (1982) immunized ramlambs 3 times, 6 weeks apart, which resulted in a significant reduction in testis size from 13 weeks onwards, while sperm motility was affected in week 24 to 26. Stanbacher (1982) immunized young ramlambs twice, resulting in castrate-like testosterone levels and 80% reduction in testis weight at 6 weeks after the second vaccination. Growth performance and carcass traits were equally affected by GnRH immunization and surgical castration. In two recent studies, single vaccination protocols were evaluated. Chinese Tanyang ramlambs immunized at 3 months of age with a GnRH-BSA conjugate in CFA showed suppressed serum testosterone and testis growth for 4 months, while the onset of sexual behavior was delayed (Cui et al., 2003). Kiyma et al. (2000) reported undetectable testosterone levels in ramlambs immunized with a GnRH-KLH conjugate in CFA at an initial body weight of 33 kg. Sexual behavior of the immunized lambs was similar to castrates, as were growth performance and carcass characteristics. Immunization with the conjugate in ISA, also reduced testosterone levels, but levels remained higher than in CFA treated animals.
Two immunizations with the ´boar taint vaccine´ Improvac, given 3 weeks apart, suppressed testosterone in ramlambs (Janett et al., 2003). However, 8 out of 10 treated animals showed an increase in testosterone between 3 and 7 months after immunization.
In adult male goats the effects of the ‘cattle vaccine’ Vaxstrate was studied (Godfrey et al., 1996). Two subcutaneous injections given either 2 or 4 weeks apart resulted in reduced levels of LH, FSH and testosterone, which decreased more rapidly in the animals that received fortnightly injections. Semen characteristics were affected: sperm motility was affected at day 112, 175 and 329, sperm counts were reduced at day 175 and 329, while ejaculate volume was only reduced at day 329 after the first immunization. Buck odour score, which was only determined until 8 weeks after the first injection, was reduced between 6 and 8 weeks in the animals vaccinated 2 weeks apart. Aggressive behavior was reduced in both vaccinated groups compared to intact male goats.

3.5 Pets

Pet overpopulation is a big problem in many countries. Stray dogs and cats are causing damage to property, spreading diseases and attacking the public, livestock and wildlife. In

the US, every year about 20 million dogs and cats are euthanized to decrease the population of unwanted pets (Anonymous, 1993). In the Netherlands, five miljoen cats are kept by private owners. Although methods to prevent pregnancies are used, the number of unwanted cats that are discarded is still increasing. Every year ten thousands of cats are brought to shelters in the Netherlands.
Neutering is the most effective way to prevent pregnancies in pets. However, costs are high, it is laborious, aneasthesia is required, post-surgical infections may occur and it is not reversible. Alternatives have been developed for this purpose. Several progestagens are on the market, which are effective in suppression of estrous in female cats and dogs by inhibition of gonadotropin release (Concannon and Meyers-Wallen, 1991). However, frequent administration is neccesary and undesirable side effects may occur like uterine diseases, diabetes, mammary tumors, increased appetite and weight gain.
The effects of GnRH immunization on fertility and sexual behavior in pets have only been evaluated in a limited number of experiments. In dogs, most studies have been performed in males, presumably due to the long estrous interval in females.

3.5.1 Dogs

In the first study in dogs (Stanbacher et al., 1983), sexually mature males were immunized subcutaneously with 200 µg GnRH conjugate in CFA at 3 monthly intervals. Two out of 5 dogs responded with reduced LH and testosterone levels while testes weights were reduced by 70%. Similar results were observed by Gonzalez et al. (1989), while others showed a response in 2 out of 2 young beagles which were intradermally treated with a GnRH-tuberculine protein conjugate (Bailie et al., 1989)
Ladd et al. (1994) performed an extensive study with GnRH conjugated to TT in Pluronic block polymer L121/MDP analog adjuvant. Five sexually mature male dogs were immunized at 0, 2 and 4 weeks. After the second immunization serum testosterone concentrations were reduced to castrate levels and maintained low for 28 weeks. At that time antibody titers had declined to pre-immune levels, resulting in a rise in testosterone levels.
A single immunization protocol using GnRH-BSA in Titermax adjuvant or Alum, did cause a decrease in testes size, especially in the Titermax group, but testosterone levels were only reduced for 2 weeks (Velasco-Moncada et al., 2000). The lack of efficacy using Alum in dogs was also reported by Ferro et al. (2004) who used a GnRH-TT conjugate and Finstadt et al. (2004) who used a synthetic GnRH antigen comprising the GnRH decapeptide and T cell epitopes. However, the latter antigen in an oil-in-water emulsion (Emulsigen combined with DDA) was effective. Testosterone was reduced to castrate levels after 3 intramuscular injections until the end of the study, 14 weeks after the third immunization.

Singh (1985) immunized 10 cyclic female dogs 4 or 5 times within 26 weeks with a GnRH-TT conjugate in CFA, MDP/Alum or LPS/Alum. Estrous was blocked in all dogs, except 1 of the 4 animals treated with the MDP adjuvanted vaccine. Gonzalez et al. (1989), demonstrated that intramuscular administration of a GnRH-KLH conjugate in squalane combined with MDP resulted in a higher GnRH antibody titer than subcutaneous administration. The latter route of administration resulted in estrous in all bitches, while in intramuscularly treated animals estrous was prevented in 5 out of 8 dogs.

3.5.2 Cats

Ladd et al. (1994) observed differences between species in their response to GnRH immunization; the vaccine that was fully effective in dogs appeared to be only partially effective in male cats. Enright and Swift (1995) reported a study in which 10-month-old male cats were immunized 4 times during a period of 12 weeks with a GnRH-OVA conjugate in DEAE-Dextran. At week 20, testosterone levels and testis size were reduced and spermatogenesis was completely suppressed. In a recent study, a single immunization with a GnRH-KLH conjugate in a novel oil-based adjuvant called AdjuVac^{TM}, containing Mycobacterium avium, reduced testosterone levels to zero for at least 4 months in 6 out of 9 one-year-old male cats (Levy et al., 2004). Robbins et al. (2004) immunized 2-month-old male and female cats by subcutaneous injections of 0.25 ml vaccine given at 2 occasions 4 weeks apart. The antigen, a recombinant leukotoxin-GnRH fusion protein was administered in Emulsigen combined with DDA. Immunization suppressed testosterone concentrations untill day 300 of the study in 4 out of 4 male cats, while all 10 immunized females were in anestrous as determined by low progesterone levels untill day 600 of the study. After immunization, tissue reactions were observed at the injection site, but the majority of the reactions had disappeared by day 28.

3.6 Wildlife species

In wildlife species, GnRH vaccination may offer a tool for population size control. In white-tailed deer immunization with a GnRH-KLH conjugate in CFA led to reduced estrous behavior, while fawning rates were reduced by 88%. Infertility lasted for up to two years (Millar et al., 2000). Another study in female white-tailed deer showed that a GnRH-OVA conjugate in DEAE Dextran did not disrupt estrous cycle (Becker et al., 1999). The authors suggested that the lack of efficacy may be due to the applied carrier protein or the timing of immunization with respect to the phase of the reproductive season.
In male red deer, immunization against GnRH prevented a reduction in feed intake and growth during the rut period and resulted in heavier animals as compared to untreated controls, as a result of the temporary suppression of testosterone levels (Freudenberger et

al., 1991). Furthermore, successfully immunized males casted their antlers prematurely during autumn instead of during spring (Lincoln et al., 1982).
Jolly et al. (1997) studied the effects of GnRH immunization on social behavior in brushtail possums, which is a host species of bovine tuberculosis in Australia. Social hierarchies remained unchanged after vaccination of dominant females.
We showed that an aqueous GnRH vaccine, as described in Chapter 7 of this thesis, caused a suppression in testosterone concentrations in young zoo-animals (blackbucks, springbok antelopes and goat bucks), while hormone levels were not affected when an adult blackbuck and springbok antelope were vaccinated (Turkstra et al., 2001). In sexually mature elephants, however, the vaccine caused a reduction in gonadal steroid production as determined by reduced faecal epiandrosterone concentrations and the bulls did not show aggressive behavior anymore (Bertschinger et al., 2004).
A single-injection programme with the GnRH vaccine GonaCon™, has been developed by the National Wildlife Research Centre in the USA to control population size of wildlife species (Levy et al., 2004). The vaccine, which contains a GnRH peptide conjugated to KLH combined with AdjuVac™ adjuvant is in the process of approval by the FDA.

3.6 Humans

3.6.1 Male contraceptive

In the past, several research groups focussed on GnRH immunization as a means for non-surgical male contraception. In order to maintain libido after GnRH immunization, exogenous testosterone had to be administered. Awoniyi et al. (1993) described a model for male contraception using GnRH immunization against GnRH in combination with small testosterone implants in rats. Low doses exogenous testosterone maintained libido in GnRH-immunized azoospermic rats. Similar results were found by Ladd et al. (1988); low doses of testosterone maintained copulation behavior and ejaculation in rats and rabbits, but the animals were not fertile. Awoniyi et al. (1992) showed that high doses testosterone are capable of restoring spermatogenesis qualitatively in adult azoospermic rats while serum LH and FSH remained undetectable. Moreover, animals were fertile as determined by the production of litters of normal size.
The vaccine used in the study of Ladd et al. (1988) was evaluated in a Phase 1 Safety study in male volunteers. The immunizations were well tolerated, but responses were variable; some men responded immediately to the immunization, while others showed a late response or did not respond at all (www.popcouncil.org). Because of the inconsistent response, further development of this vaccine was stopped.
GnRH immunization combined with testosterone supplementation resulting in infertility with maintenance of libido, could be a suitable contraceptive in men. However, besides the inconsistent responses, several factors have hampered the application of GnRH

immunization as a male contraceptive. For instance, the availability of several appropriate contraceptive methods and the expected difficulties getting such a vaccine approved for healthy humans, considering the side effects of an adjuvanted vaccine which has to be administered on a regular basis.

3.6.2 Anticancer therapy

As the GnRH vaccine effectively reduces gonadal steroid levels, it could be a promising tool to treat gonadal steroid-dependent diseases such as prostate cancer, breast cancer and endometriosis. Here, we focus on prostate cancer.

3.6.2.1 Prostate cancer

Prostate cancer is the second most common type of cancer in men. Worldwide more than 650.000 new cases are diagnosed every year of which about 190.000 in the US and 190.000 in the EU including 6000 in the Netherlands.The incidence rates are severely influenced by the availability of testing in a population. For instance, the incidence in the USA is twice as high as in most European countries, due to PSA testing in the USA. In Europe, an estimated 30% of the diagnosed men dies from the disease (www.afud.org/education/prostate/prostatecancer.asp).

Treatment of prostate cancer depends on the age of the patient and the stage and the growth rate of the tumor. In older men with slow growing tumors no treatment is advised as side effects of the treatment may outweight the benefits. Prostatectomy is the most common treatment for early stage prostate cancer. It is a successfull therapy, but has a high risk for impotence and urine incontinence. Radiation therapy can be applied in early stage tumors as the primary therapy, in cases surgery can not be performed or after surgery to prevent recurrency of the cancer. In metastatic prostate cancer, which up to now is not curable, radiation therapy can relief the pain. Hormone therapy is mainly applied when the cancer has metastasized or to prevent recurrence after prostatectomy or radiotherapy. It blocks the release of male steroids, which are necessary for prostate tumor cell growth. Hormone therapy includes: (i) surgical castration, (ii) GnRH agonist treatment, in order to prevent the testes from producing androgens by overstimulation of the GnRH receptor, which results in a reduction in LH and FSH release by the pituitary and (iii) anti-androgens treatment, which blocks the action of androgens by occuping the androgen receptor in the tumor cells. Although commonly used, these therapies all have side effects. Surgical castration and GnRH agonists are causing impotence and loss of libido. GnRH agonist treatment also causes a so-called flare-up reaction. The agonist initially enhances testosterone production by overstimulation of the GnRH receptors, resulting in enhanced tumor growth for some weeks. Gradually, however, the treatment causes testosterone levels to drop to very low levels. For this reason GnRH agonist treatment can not be used as mono therapy in patients

with spinal cord metastasis as nerve compression may occur. Anti-androgens, which are given to avoid the flare-up reaction or as combination therapy with GnRH agonists, are causing nausea, vomiting and diarrhea, whereas long term treatment may cause liver problems as well as gastrointestinal side effects.
GnRH vaccination could be an alternative for hormone therapy, as it reduces testosterone to undetectable levels, without inducing a flare-up. This also implicates that GnRH vaccination can be applied in patients with spinal cord metastasis without the use of anti-androgens to counteract the flare-up response. Other advantages of vaccination against GnRH as compared to the commonly used GnRH agonist therapy, could be the lower frequency of administration and lower costs of the treatment.

3.6.2.2 Clinical trails
Several GnRH vaccines have been developed for the treatment of prostate cancer. The group of Talwar used a vaccine comprising a modified GnRH decapeptide with a D-Lysine at position 6 linked to DT. This vaccine reduced testosterone concentrations to castrate levels and caused atrophy of the reproductive organs in rats and monkeys (Jayashankar et al., 1989; Giri et al., 1990; Giri et al., 1991; Furst et al., 1994). Fuerst et al. (1997) studied the vaccine in rats bearing androgen-dependent prostatic tumors (R3327-PAP). These so-called Dunning tumor cells are implanted under the skin and develop into a measurable tumor. After three monthly immunizations tumor growth was suppressed compared to untreated controls. Surprisingly, tumor growth was also suppressed in rats implanted with androgen-independent Dunning tumor cells (R3327-AT2.1). The authors suggest that a local GnRH-loop exist in the prostate, which is affected by GnRH neutralizing antibodies, causing a reduction in tumor growth even in testosterone-independent tumors.
Acute and chronic toxicology studies with this vaccine have been performed in rats and monkeys. Three intramuscular injections given at monthly intervals, showed no detrimental effects, whereas testosterone levels declined in all treated animals (Gual et al., 1997). Clinical trials in patients with advanced prostate cancer revealed that in contrast to rodents and monkeys, high antibody titers were obtained in some, but not all treated patients. A reduction in prostatic size was observed in 3 out of 6 patients treated with 400 µg conjugate in Alum and in 1 out of 6 patients treated with 200 µg conjugate (Talwar et al., 1995). The vaccine was also evaluated in postmenopausal women with elevated LH and FSH levels due to severe hypoestrogenism. After 2 injections with 300 µg GnRH equivalent of the conjugate in Alum, LH and FSH levels were decreased for 4 months in all 3 patients (Gaul et al., 1997).
Recently, the group of Talwar developed a recombinant GnRH vaccine (Talwar et al., 2004). Reason for this change in type of antigen, were the high costs for the synthesis of their GnRH-DT conjugate, possible difficulties with the reproducibility when manufactured

on a large scale, and the occurence of carrier-induced immune suppression in some treated subjects due to the use of DT (Gaur et al., 1990). The recombinant product consists of 6 GnRH repeats and five different promiscue T-cel epitopes. Multiple vaccination with CFA as the adjuvant resulted in significantly reduced testes and accessory sex gland in all treated rats.

The GnRH antigen developed by the company Aphton (Wodland, USA) comprises the GnRH molecule extended with a linker peptide of 6 amino acids conjugated to DT. In their first study, 12 men with advanced prostate cancer were vaccinated with 30 or 100 µg conjugate in a water-in-oil emulsion (Simms et al., 2000). Four patients, 2 of each dose group, responded with a reduction in testosterone and PSA levels for 70 to 250 days. Three and 15 µg doses were evaluated in order to determine the minimal effective dose (Parkinson et al., 2004). Suppression of testosterone to castrate levels was detected in 2 out of 6 patients treated with 15 µg antigen, whereas none of the patients treated with 3 µg responded. Antibodies raised against the Aphton GnRH vaccine have also been tested in an animal model for human breast cancer. Passive immunization with purified antibodies raised in rabbits inhibited growth of oestrogen-sensitive MCF7 human breast cancer tumors in nude mice (Jacobs et al., 1999).

The Population Council in New York, who first focussed on an antifertility vaccine for men, studied their vaccine in rats bearing androgen-sensitive prostate tumors (Ladd et al., 1995). The vaccine, which contains a native GnRH molecule with glutamine at position 1 for conjugation to TT, was evaluated in combination with GnRH antagonist treatment. It appeared that tumor size in the animals receiving the combined GnRH antagonist plus GnRH vaccine treatment was significantly lower compared to animals receiving a single treatment. Clinical trials in prostate cancer patients have not been performed so far.

The company Biostar, nowadays known as MetaMorphix Canada (Saskatoon, Canada), developed a GnRH vaccine comprising a recombinant fusion protein produced in E. Coli bacteria: several copies of a GnRH-tandem molecule were fused to the terminal ends of leukotoxin. This vaccine has shown full efficacy in young pigs and cats (Manns and Robbins, 1997; Robbins et al., 2004), while antibody responses were variable in heifers (Cook et al., 2000). For application in prostate cancer patients, the vaccine called Norelin™, was out-licensed to York Medical BioSciences (Mississauga, Canada). In 2001 clinical studies indicated that the vaccine with an aluminium salt-based adjuvant was safe to be used in humans, however it was not immunogenic enough to raise a sufficiently strong immune response. In 2003, a second clinic trial was initiated. This vaccine was well tolerated with ´no major adverse events´. Immune responses could be determined in 60% of the patients (www.ymbiosciences.com).

United Biomedical (UBI, Hauppauge, USA) developed a complete synthetic vaccine comprising the GnRH decapeptide and several promiscue T-cel epitopes. Immunogenicity

of the GnRH-T cell epitope constructs was enhanced by addition of a domain from Yersinia invasin protein (Finstad et al., 2004). Although the single constructs were not completely effective in rats, mixtures of constructs caused serum testosterone to drop to very low levels, whereas testes weights were less than 25% of the controls. The antigens in a water-in-oil formulation and oil-in-water formulation were effective in baboons and dogs, respectively. Furthermore, androgen-responsive Dunning prostate tumor cell growth was reduced as a result of vaccination. Three initial vaccinations followed by two boosters several month later, blocked tumor growth for more than 60 weeks. Phase 1 clinical trials have been performed and follow-up trials were planned for 1999, however, results have not been published so far. In a recent press release UBI announced trials in prostate cancer patients in China (www.unitedbiomedical.com)
Proterics (formerly known as Proteus) developed a GnRH vaccine ´Prolog´, which was out-licensed to ML Laboratories. They completed phase II clinical studies in 2000, but at present no results have been published (www.mllabs.co.uk). The vaccine containing the GnRH decapeptide with an additional glycine and cysteine has been tested with variable results in several animal species (Enright and Swift, 1995; Finnerty et al., 1996; Becker et al., 1999).

4. CONCLUSION

A considerable amount of research has been performed in order to develop GnRH vaccines. Several vaccines have proven efficacy in experimental studies in a variety of species and applications. Sofar, however, no vaccine has been satisfactory applied in the target species. Most vaccines gave variable or short term responses, while effective vaccines suffered from adverse effects due to use of harsh adjuvants. For applications that require long term and full efficacy without adverse effects, e.g. fertility control in pets, more research is needed. For other applications, however, a lesser degree of efficacy is acceptable, e.g. population size control in wild life species. A few GnRH vaccines are already on the market. It is expected that more efficient vaccines will be commercialized in the near future.

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Chapter 3

New GnRH-like peptide construct to optimize efficient immunocastration of male pigs by immunoneutralization of GnRH

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ABSTRACT
Castration of male pigs is routinely performed in order to prevent the occurrence of boar taint in pig carcasses. However, boar taint can be eliminated also by immunological castration using a synthetic peptide vaccine against GnRH. For pig farming, to make immunocastration a feasible alternative method to surgical castration the composition of the vaccine has to be not only reliable and effective but also cost-efficient and safe. Previously we have developed an effective immunocastration vaccine by replacing the monomer GnRH by a much more immunogenic tandem peptide. However, this tandem-GnRH vaccine preparation needs Complete Freund's adjuvant and to be applied at a relatively high dose. Therefore alternative antigens were designed to cope with this problem and tested with different adjuvants and dosages. An effective new antigen was designed based on a GnRH-tandem peptide, which was dimerized and modified in one amino acid position of the decapeptide to allow conjugation of this tandem-dimer to ovalbumin. In mild adjuvants and in low dosage this antigen was very effective in reducing testis weight, serum LH and androstenone levels in backfat. Thus an improved immunocastration vaccine has been designed that is relatively cost-efficient and highly efficacious in two vaccinations at low dose.

1. INTRODUCTION

Surgical castration of young male pigs is common practice in pig farming today. Castration is performed to prevent the occurrence of pork with a bad odor ("boar taint") which originates mainly from the male sexual organs (1-9). However, surgical castration is economically undesirable, as it leads to growth retardation, reduced carcass quality and higher farming costs due to more inefficient feed conversion (10-12). In addition there is increased susceptibility to diseases (13), and pain stress due to the surgery without anesthesia (14). To circumvent the disadvantages of surgical castration immunocastration by neutralizing GnRH seems the best feasible option (15). Immunocastration is achieved by immunizing boars against gonadotropin releasing hormone (GnRH) to disrupt the hypothalamic-pituitary-gonad axis, thereby effectively inhibiting testis growth and synthesis of male sexual steroid hormones. One testicular steroid produced is testosterone, which is responsible for the efficient feed conversion and lean meat production of boars, in addition to the male sex characteristics. Another testicular steroid is androstenone, which is the main agent responsible for boar taint (1-9). Ideally, it is desirable to keep testosterone present as long as possible, while at the same time androstenone has to be completely absent at the time of slaughter. In addition to androstenone, skatole (3-methyl-indole) appears to be another compound contributing to boar taint. In pigs slaughtered at a low weight (± 80 kg) this compound may contribute more to boar taint than androstenone (16, 17). However, whether skatole determination (18) suffices as boar taint parameter in other pig genotypes and at heavier slaughterweights than commonly used in Denmark is questionable (19). Although this compound also renders an offensive odour to pork it is not entirely male specific and its contribution can be controlled for a great deal by management factors such as cleanliness, and especially diet composition (20).

Immunisations against GnRH to immunocastrate pigs have been performed using various conjugates of native GnRH. Often multiple injections are administered, using CFA or other adjuvants. Results vary widely in individual animals, meaning that no consistent response was elicited (21-23). Recently, good results were obtained when the second immunization was postponed to a few weeks before slaughter, showing low androstenone levels in combination with a boar-like economic performance (24). However, additional on-site testing for low androstenone levels remains needed in this case as these immunocastrates cannot otherwise be distinguished from intact boars.

Initial immunocastration experiments using a GnRH-tandem-KLH conjugate in CFA administered at 10 and 18 weeks of age were completely successful in reducing testis size and weight, together with reduction of androstenone to a low level in boars (25). Experiments were performed with a variety of different GnRH-vaccine formulas, which yielded a high variability in successrate. A strong correlation appeared to exist between testis size and weight and the presence of androstenone in backfat (26). The level of andros-

tenone in backfat is a proper parameter for boar taint in Dutch circumstances, using commercial mixed breeds at relatively heavy slaughter weights (17, 27). Testis size or weight thus could serve as a convenient parameter for the absence of boar taint, after immunizations at 10 and 18 weeks of age (26), making evaluation of successful immunocastration possible by visual inspection just before slaughter.
Several aspects necessarily need to be taken into account in the development of an immunocastration vaccine. This paper describes the optimization of an immunocastration vaccine with respect to three variables. First, the antigen has to be effective in preventing boar taint in immunocastrated pigs. Second, the vaccine and thus the individual components thereof need to be producable at reasonable costs to allow an attractive sales price to the pig farmer. Third, all components of the vaccine have to be safe and acceptable for use in consumption animals. Our experiments indicate that immunocastration of pigs using an optimized vaccine comprising a modified GnRH-tandem-dimer-ovalbumin conjugate in Specol is a practically feasible alternative for surgical castration in pigs.

2. MATERIALS AND METHODS

2.1 Peptide synthesis

The synthesis of GnRH and the Tandem-GnRH peptide has been described (25). Peptide syntheses were performed on an ABI 430A peptide synthesizer using FastMoc cycles on a 0.25 mmole scale with cycle times of about 60 min (28, 29). Peptide purification was carried out using a Waters PrepLC4000 system,equipped with Waters PrepPak Cartridges (25 mm x 210 mm or 40 mm x 210 mm) filled with Delta-Pak C18 (15 μm, 100A) material. For analytical HPLC, two Waters pumps model 510, a Waters gradient controller model 680, a Waters autoinjector model WISP 712, and a Waters photodiode array detector model 991 were used. The products were analyzed in a linear gradient from water with 0.1% trifluoroacetic acid (TFA) to 60% acetonitrile in water with 0.1% TFA in 60 min on a Waters Delta Pak C18-100A (3.9 x 150 mm, 5 μm) column at 1 ml/min at 215 nm. All products were >95% pure according to the peak area. Amino acid analysis was done with a Waters PicoTag system. The results were in agreement with the expected values according to the amino acid sequences. Peptide sequences synthesized were, in single letter amino acid code:

GnRH(C):	<QHWSYGLRPGC#;
Tandem-GnRH:	<QHWSYGLRPGQHWSYGLRPGC#;
Tandem-GnRH (N-term. Cys):	*CQHWSYGLRPGQHWSYGLRPG#;
<Q1C,H2K-GnRH:	CKWSYGLRPG# (30);
GnRH(4-10):	SYGLRPG# (31);

GnRH(1-7):	<QHWSYGL# (31);
G6Naf-GnRH:	<QHWSYNafLRPGC#;
G6Naf-GnRH-tandem:	<QHWSYNafLRPGQHWSYNafLRPGC#;
G6k-GnRH:	<QHWSYkLRPGC#;
G6k-GnRH-tandem:	<QHWSYkLRPGQHWSYkLRPGC#;

<Q = pyroglutamic acid (pyroGlu); * = acetyl; # = amide; <Q1C,H2K = pyroGlu at position 1 replaced by Cys and His at position 2 replaced by Lys (30); Naf = 2-naphtyl-D-Ala; G6k = Gly at position 6 replaced by D-Lys; k = D-lysine.

2.2 Dimerization procedure

HPLC-purified product was dimerized by dissolving the product in 20% DMSO in water. Dimerization was more than 95% efficient. The pH was adjusted to 5 - 6 with 1% or 2% NH_4HCO_3, maintaining a clear solution. Correction of pH was done with 1-10% acetic acid. After stirring at room temperature for at least 5 h, the product was purified directly using HPLC. Separation was complete at baseline with 2 minutes difference on analytical HPLC.

2.3 Conjugate preparation

Conjugation to Keyhole Limpet Hemocyanin (KLH) (Tandem-GnRH-KLH conjugate) via maleinimidobenzoyl-N-hydroxysuccinimide ester (MBS) has been described (25). For conjugation via N-ethyl-N'-(3'-dimethylaminopropyl)carbodiimide hydrochloride (EDC) to KLH (<Q1C,H2K-GnRH-dimer-KLH conjugate), to bovine serum albumin (BSA) (GnRH(4-10)-BSA conjugate and GnRH(1-7)-BSA conjugate) or to ovalbumin (G6k-tandem-dimer-OVA conjugate) an equal weight of both the peptide and the carrier protein were dissolved separately in milliQ-water and both solutions were mixed well. Next a 10-fold excess, based on weight equivalents, of EDC was dissolved in milliQ water. Subsequently this solution was slowly added to the solution of peptide/ovalbumin under continuous stirring. After at least 6 h the product was dialyzed (MW cut-off 10,000) against water. The loading was calculated from comparative amino acid analysis of the conjugate and the separate peptide and carrier protein. According to the amino acid analyses the conjugates contained approximately 0.5 mg of peptide per mg of carrier protein.

2.4 Vaccine formulation

Adjuvants tested were complete Freund's adjuvant (CFA), incomplete Freund's adjuvant (IFA), double oil emulsion and Specol. CFA or IFA was mixed 1:1 with the peptide solution to a stable emulsion (25). Double oil emulsion (ID-DLO; W/O/W 10:11:10 v/v) consisted of a water phase (antigen in PBS (Phosphate Buffered Saline), an oil phase (light mineral oil with emulgator) and a second water phase (PBS with detergent). Specol

(Special Oil Phase; ID-DLO) consisted of two detergents in a light mineral oil. For preparation of emulsions in Specol, 4 parts of the water phase containing the antigen were mixed with 5 parts Specol (v/v) (32, 33).

2.5 Animals

Pigs of commercial mixed breeds have been used throughout all experiments. Pigs were housed at the pig management facilities of the Veterinary Faculty, University of Utrecht, at the ID-DLO experimental farm Bantham, Maartensdijk and at a commercial pig farm and kept according to the general pig management rules.

2.6 Immunization protocol

Each animal received 1 mg of peptide or 1-1.5 mg of conjugate, or a lower amount as indicated, prepared as described above. Intact male pigs were approximately 10 weeks of age at the start of the experiment, when they received the first immunization (day 0). The booster administered 8 weeks after the first immunization (8 wpv) had the same composition. When CFA was used, the second immunization was administered in IFA. All other adjuvants were used for both immunizations. The vaccine was administered intramuscularly in the neck. All animals were slaughtered 8 weeks after the second vaccination (16 wpv). Control groups of boars were mock immunized with carrier and adjuvant or left untreated. Data were combined as no differences were seen between the different control groups.

2.7 Evaluation parameters

2.7.1 Testis size and blood samples during the trial

Testicle sizes were measured externally using calipers at the first immunization (day 0) and every four weeks thereafter (4, 8, 12 wpv), until just before slaughter (16 weeks after first immunization, 16 wpv). At these times also serum samples were taken by vena jugular puncture for determination of porcine LH and antibodies against GnRH.

2.7.2 Testis weight and backfat samples after slaughter

After slaughter testicles were excised, epididymes were removed and testes weight was recorded. Samples of backfat were taken for androstenone determination and kept at -20°C.

2.8 Assays

2.8.1 Antibody titers

Amount of antibodies to GnRH was determined as described in ref. 25 by binding of serial dilutions of the pig antisera to ^{125}I-GnRH. Titers are expressed as percentage binding of ^{125}I-GnRH at a given serum dilution.

2.8.2 Androstenone

The amount of androstenone in backfat was determined using an androstenone-Elisa kit (34) from R-Biopharm, Darmstad, Germany and purchased from Ridascreen, Almere, The Netherlands. Sensitivity of the assay is 0.05 μg androstenone/g backfat, and the coefficients of variation were 13% and 16%, for intraassay and interassay variability, respectively.

2.8.3 Porcine LH

A radioimmunoassay was used (35). pLH for iodination was also used as assay reference preparation and was from UCB (H028/H LH (porcine). The antiserum was the same as in ref. 35. Validation of this assay was performed as described (35). Interassay variability was 9.14% and intraassay variability was 10.6%.

2.9 Statistics

To test if sample differences were statistically significant the Mann-Whitney (or Wilcoxon) U-test was used. Significance is indicated by p-value (<0.01 or <0.05).

3. RESULTS

3.1 Control animals

In the experiments sham-immunized or untreated boars were included as controls. The results of all control groups were not different and therefore were pooled. In 125 boars the median testis weight at 26 weeks of age was 231 g (range 65-447 g) (Table 1).

Table 1. Effectivity of Tandem-GnRH vaccines at a 1 mg dose in CFA/IFA.

	No. of responders per total no. (% success)	Testis weight (g), Median (n; range) responders only	pLH (ng/ml) at 16 wpv or 26 weeks of age median (n; range), responders only
Controls	n.a.	231 (125; 65-447)	0.85 (69; 0.38-2.1)
Tandem-GnRH-KLH conjugate	41/43 (95)	46 (41; 10-143)	--
Tandem-GnRH + KLH, not conjugated	21/25 (84)	30 (21; 9-123)	--
Tandem-GnRH, no carrier protein	133/137 (97)	24 (133; 7-132)	0.52 (107; 0.25-1.37)

Vaccines were prepared and administered and results evaluated as described in Material and Methods. Responders were defined as pigs with a testis weight of <150 g (26), except the control group that includes all animals. Serum levels of pLH were determined as described in Material and Methods.

N.a., not applicable. --, not determined.

In 62 of these boars androstenone levels have been determined and the median level was 0.81 µg/g fat (range 0.07-6.8). The proportion of this group that had androstenone levels higher than 0.5 µg/g was 77% (48 out of 62) (data not shown). Serum levels of pLH measured in 389 pigs at the age of 10 weeks, i.e. the total of control boars and experimental pigs before the first immunization (day 0), ranged from 0.37-6.65 ng/ml with a median level of 1.14 ng/ml (data not shown). In contrast, in a group of 16 barrows, surgically castrated in the first week of age, median pLH level was higher than in control or treated animals, both at day 0/10 weeks of age: 3.86 ng/ml (range 1.7-7.14) ($p<0.01$ compared to intact animals of the same age) and at 26 weeks of age: 2.29 ng/ml (range 0.9-3.21) ($p<0.01$ compared to 26 week old boars) (data not shown). The relatively high pLH levels in barrows most probably reflect the lack of negative feedback by testicular steroids on pituitary LH secretion. In 69 boars just before slaughter at 26 weeks of age median pLH level was 0.85 ng/ml (range 0.38-2.1) (Table1).

3.2 Effectiveness of vaccines based on the Tandem-GnRH peptide

A dose of 1 mg of Tandem-GnRH-KLH vaccine administered twice at 10 (day 0) and 18 weeks of age (8 wpv) was very effective in inhibiting testicular development (25). Several experiments using groups of 7-10 pigs per treatment showed that effectivity was high, as 41 out of 43 animals had small testes at slaughter (Table 1). Successful immunocastration was defined as a final testis weight of less than 150 g after immunization (26). Tandem-GnRH vaccine administered together with, but not conjugated to, the carrier protein was successful in 21 out of 25 pigs (Table 1). Surprisingly, Tandem-GnRH only, unconjugated to a carrier, also appeared to be very effective resulting in testis weights of less than 150 g in 133 out of 137 pigs (Table 1). Similarly to our previous experience, pigs with testis weights lower than 150 g had androstenone levels in backfat of much less than 0.5 µg/g (median 0.03, range 0.01-0.34, n=69) ($p<0.01$ compared to androstenone levels in intact boars; see above) (data not shown). Serum levels of pLH at day 0 (10 weeks of age) were 1.14 (0.37-6.65) ng/ml (median and range, n=389), as mentioned above. In the immunocastrated pigs at 16 wpv (26 weeks of age) median pLH level were reduced to 0.52 (range 0.25-1.37; n=107) (Table 1), which is significantly ($p<0.01$) different from pLH levels in 26 week old control boars and from pLH levels at day 0.

However, this high effectivity in reducing testis weights only held true for high doses of antigen administered in CFA. Either lowering of the antigen dose to 100 µg or changing the adjuvant administration from CFA/IFA to IFA/IFA strongly compromised the effectiveness of immunocastration measured at the level of testes weight and pLH levels (Table 2). Only in immunized animals with testis weights <150 g serum LH levels at 16 wpv were reduced compared to non-responders ($p<0.01$), controls ($p<0.01$) and day 0 levels ($p<0.01$), whereas

in non-responders pLH levels were comparable to those of control boars (p=0.69) and had not dropped in time compared to 10 weeks of age/day 0 (p=0.08) (Table 2).

Table 2. Effectivity of Tandem-GnRH peptide vaccine. Effect of dosis and adjuvant.

Dosis	Adjuvant	Testis weight (g), responders, Median (n; range)	Testis weight (g), non-resp. Median (n; range)	pLH (ng/ml) at 16 wpv, responders, Median (n; range)	pLH (ng/ml) at 16 wpv, non-resp., Median (n; range)
1 mg	CFA/IFA	39 (11; 23-69)	--	0.48 (11; 0.26-0.81)	--
1 mg	IFA/IFA	20 (5; 18-87)	226 (8; 189-318)	0.45 (5; 0.27-0.58)	0.94 (8; 0.61-1.69)
0.1 mg	CFA/IFA	58.5 (7; 13-154)	213 (5; 180-306)	0.5 (7; 0.29-0.74)	0.93 (5; 0.69-0.98)
0.1 mg	IFA/IFA	68 (5; 23-153)	242 (8; 186-300)	0.53 (5; 0.36-1.38)	1.07 (8; 0.51-1.4)

Vaccines were prepared and administered and results evaluated as described in Material and Methods. Responders were defined as pigs with a testis weight of <150g (26). Serum levels of pLH were determined as described in Material and Methods.

3.3 Effectiveness of other vaccines based on GnRH

As other peptide constructs based on GnRH had been claimed to be effective immunocastration agents (30, 31), we tested three of these compounds under our experimental conditions. In two doses of 1 mg antigen-conjugate and with CFA/IFA as adjuvant these constructs indeed appeared to be effective in suppressing testicular development. <Q1C,H2K-GnRH-dimer-KLH conjugate (30) was effective in 9 out of 9 pigs and final testis weight was 31 ± 8 g (mean ± s.d.). GnRH(4-10)-BSA conjugate (31) was effective in 6 out of 7 animals with a testis weight of the 6 responders being 30 ± 9 g (mean ± s.d.). GnRH(1-7)-BSA (31) was much less effective. Only 3 pigs out of 8 had testis weighing less than 150 g (data not shown).

3.4 Effectiveness of different antigen constructs in milder adjuvant

Due to its negative connotations the use of CFA for the first immunization was abandoned and replaced by IFA. Although Tandem-GnRH in unconjugated form was less effective in IFA/IFA (see also Table 2), the dimerized form, Tandem-GnRH-dimer vaccine, was able to immunocastrate 5 out of 7 pigs. Immunocastration is defined as having a testis weight of less than 150 g at slaughter (26 weeks of age), after immunization (Table 3). Immunizations with a number of different monomer, dimer, tandem and tandem-dimer GnRH-like

formulas (Figure 1) using IFA as adjuvant both at first and second immunization showed that in an adjuvant milder than CFA only a few unconjugated peptide constructs were effective in inhibiting testis growth in pigs to a weight of less than 150 g (Table 3).

Table 3. Effect on testis weight of different unconjugated antigens at 1 mg dose in IFA/IFA adjuvant. The effect of tandem-formulation and dimerization.

Antigen	No. of responders per total no.	Testis weight (g), Median (range), responders	Testis weight (g), Median (range), non-resp.	pLH (ng/ml) at 16 wpv, Median (range), responders	pLH (ng/ml) at 16 wpv, Median (range), non-resp.
GnRH(C)	0/7	--	249 (152-377)	--	n.d.
GnRH-dimer	0/7	--	304 (209-345)	--	0.79 (0.7-1.01)
Tandem-GnRH	2/7	80, 83	289 (180-318)	n.d.	n.d.
Tandem-GnRH-dimer	5/7	17 (16-86)	225, 255	0.55 (0.47-0.66)	0.79, 1.12
Tandem-GnRH (N-term. Cys)	2/7	74, 92	206 (186-265)	n.d.	n.d.
Tandem-GnRH-dimer (N-term. Cys)	12/15	39 (11-141)	194 (193-236)	0.45 (0.35-1.26)	1.07 (0.66-1.8)
<Q1C,H2K-GnRH	0/7	--	289 (171-332)	--	n.d.
<Q1C,H2K-GnRH-dimer	1/7	81	239 (170-299)	n.d.	n.d.
GnRH(4-10)	1/7	128	270 (206-283)	n.d.	n.d.
GnRH(4-10)-dimer	0/7	--	250 (175-318)	--	n.d.
G6Naf-GnRH-dimer	2/7	60, 131	180 (176-263)	0.7, 0.8	0.73 (0.65-1.24)
G6Naf-GnRH-tandem-dimer	0/7	--	256 (177-322)	--	1.02 (0.59-1.99)
G6k-GnRH-dimer	2/7	138, 139	222 (166-248)	0.59, 1.2	0.64 (0.57-1.62)
G6k-GnRH-tandem-dimer	5/7	46 (29-56)	225, 275	0.52 (0.47-0.61)	0.63, 1.29

Peptides were synthesized, vaccines prepared and administered and results evaluated as described in Material and Methods. Responders were defined as pigs with a testis weight of <150g (26). Serum levels of pLH were determined as described in Material and Methods.
N.d., not determined. --, not applicable.

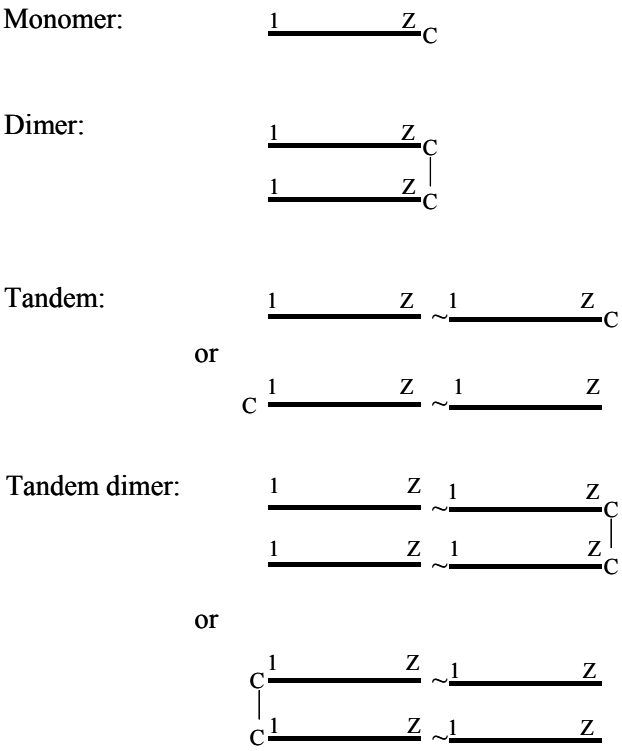

Figure 1. Schematic illustration of the different antigen configurations. 1-Z: The amino acid number in the basic peptide, e.g. for GnRH Z=10. C: carboxy- or amino-terminal cysteine residue which can be used in monomer and tandem configurations to conjugate the antigen to a carrier molecule or in dimer and tandem-dimer configurations to dimerize monomer or tandem subunits. ~: A direct link between Z and 1.

GnRH-C, its dimerized form, Tandem-GnRH with a cysteine at the carboxy- or amino-terminal end of the peptide, the constructs from refs. 30 and 31 mentioned above viz. <Q1C,H2K-GnRH and GnRH(4-10), both as monomer and dimer, G6Naf-GnRH (the GnRH-analog Nafarelin) as dimer and tandem-dimer all were incapable of immunocastrating pigs effectively. Only three constructs were effective to an approximately equal extent. First, Tandem-GnRH-dimer, dimerized via the carboxy-terminal cysteine, was already mentioned above to be effective in 5 out of 7 pigs. Second, Tandem-GnRH-dimer, dimerized via the amino-terminal cysteine, was effective in 12 out of 15 pigs. We next considered that conjugation to a bulky carrier probably would improve effectiveness. As the tandem-dimer formulas in the form of Tandem-GnRH-dimer

dimerized via cysteine at the carboxy- or amino-terminal end of the peptide could not be conjugated, we changed one amino acid to allow for conjugation. The dimer-form of GnRH with D-lysine (k) replacing L-glycine (G) at position 6 (G6k-GnRH-dimer) was not effective but the G6k-GnRH-tandem-dimer formula in its unconjugated form was the third construct, that was as effective as the C- or N-terminally dimerized Tandem-GnRH-dimer. G6k-GnRH-tandem-dimer immunization immunocastrated 5 out of 7 pigs (Table 3). Apparently the tandem principle is very essential to obtain an effective immunocastration vaccine, and dimerization yields an even higher effectivity to these constructs. However, even these three tandem-dimer formulas were not fully effective, that is, did not immunocastrate all immunized pigs, when administered in unconjugated form in an adjuvant that is milder than CFA (Table 3). The levels of pLH at 16wpv in the responders of the three most effective treatments were significantly lower compared to control boars ($p<0.01$), while the non-responders in these groups had pLH levels not significantly different from controls ($p=0.71$).
As the G6k-GnRH-tandem-dimer allows for conjugation to a carrier substance, in contrast to both Tandem-GnRH-dimers (C- and N-), the former antigen was selected for further evaluation. Therefore, the G6k-GnRH-tandem-dimer was conjugated to ovalbumin and subsequently tested in two mild oil adjuvants developed at ID-DLO (32, 33) at a dose of 500 µg of peptide (1.5 mg of conjugate). Nine pigs received Specol and eight pigs received double oil emulsion as adjuvant. The vaccine appeared to effectively immunocastrate the treated pigs. Individual pigs had testes weights much lower than 150 g, except one pig from the double oil emulsion group (154 g) (data not shown).

3.5 Effectivity of lower doses of G6k-GnRH-tandem-dimer-ovalbumin conjugate
A dose of 500 µg of peptide (1.5 mg of conjugate), administered twice at 10 and 18 weeks of age was very effective in inhibiting testicular development. From several experiments using groups of 6-15 pigs per vaccine dose it appeared that no true dose-dependent effect existed on testis weight at slaughter (Table 4). Defining successful immunocastration as a final testis weight of less than 150 g after immunization (26), Table 4 shows that also doses as low as 5 µg could effectively reduce testis weight to the same extent as the 500 µg dose. However, in the groups receiving the lowest doses the occurrence of non-responders became obvious. In two groups receiving a high dose of 500 and 250 µg antigen also a few non-responders were seen. The bad responders from these two groups occurred in one particular experiment in which many of the pigs had general health problems, which we hypothesize might have compromised their immune status to the extent of being less capable to respond to immunization. In different experiments high as well as lower doses of antigen effectively reduced testis weight to less than 150 g in all treated pigs (Table 4). Anti-GnRH-titers determined in a 1:2000 dilution of 12 wpv antisera of successfully

immunocastrated pigs usually were higher than 20% (Table 4), whereas non-responders had titers lower than 20% (data not shown). Compared to pLH levels at day 0/10 weeks of age (median 1.14 ng/ml; range 0.37-6.65; n=389, as mentioned above) and to pLH levels at 26 weeks of age in controls (Table 1) in the immunocastrated animals these levels had dropped considerably at 16 wpv ($p<0.01$) (Table 4).

Table 4. Effectivity of different doses of G6k-GnRH-tandem-dimer-ovalbumin conjugate in Specol.

Dosis (µg), peptide content	% success (No. of responders per total no.)	Testis weight (g), Median (range), responders only	Titers[a], Median (range), responders only	pLH (ng/ml) at 16 wpv, Median (range), responders only
500	95 (16/17)	21 (11-134)	48.6 (33.9-53.8)	0.59 (0.52-0.81)
450	100 (11/11)	36 (8-120)	24.6 (15.7-29.6)	0.45 (0.24-0.74)
250	78 (7/9)	21 (16-82)	40.4 (35.7-54.8)	0.52 (0.39-0.63)
150	100 (26/26)	19.5 (11-79)	32.9 (21.4-40.2)	0.47 (0.37-0.75)
125	100 (9/9)	32 (15-50)	43.7 (29.8-56.3)	0.55 (0.40-0.63)
62.5	100 (43/43)	19 (8-140)	28.5 (20.4-54.9)	0.51 (0.39-1.34)
50	90 (9/10)	36 (18-89)	25.4 (19.8-30.4)	0.46 (0.44-0.56)
15	88 (23/26)	26 (9-74)	32.7 (20-37.4)	0.49 (0.33-0.76)
5	82 (9/11)	41 (15-108)	25.8 (22-31.5)	0.43 (0.39-0.6)
1.5	40 (6/15)	76 (13-150)	31.5 (25.1-33.6)	0.58 (0.53-1.33)

Vaccines were prepared and administered and results evaluated as described in Material and Methods. Responders were defined as pigs with a testis weight of <150g (26). Antisera titers against GnRH and serum levels of pLH were determined as described in Material and Methods.

[a] Titer was determined as % binding of ^{125}I-GnRH at 1:2000 dilution of 12 wpv antisera.

When data on pLH levels for all animals that responded to immunization at 16 wpv with testis weights of less than 150 g (n=305; median 0.51 ng/ml, range 0.24-1.38) were compared to the pLH data of pigs with testis weights higher than 150 g at 26 weeks of age/16 wpv, viz. untreated or sham-immunized boars (controls, n=69) plus all non-responding immunized pigs (n=70) (combined n=139, median 0.89 ng/ml, range 0.38-2.1), a significantly ($p<0.01$) lower median pLH level after immunocastration was observed.

4. DISCUSSION

The results described above indicate that we have been able to develop an improved immunocastration vaccine against GnRH which is practically applicable in pig farming industry.

To develop a vaccine against GnRH the low immunogenicity of the 'self'-like antigen needs to be overcome. In general immunogenicity of small antigens such as peptides needs to be increased by coupling to a large carrier molecule, and/or changing the structure of the antigen (36). Furthermore, an immunocastration vaccine for pigs needs to be tested directly in the target animal as efficacy may differ between species. The immune system of pigs apparently recognized Tandem-GnRH-KLH-conjugate as might be expected, but surprisingly also the single Tandem-GnRH molecule appeared to be sufficiently immunogenic to elicit GnRH-neutralizing antibodies in 97% of the vaccinated pigs to the extent of full immunocastration. In our hands, two out of three different antigen formulas described elsewhere (30, 31) appeared to be effective too when dimerized (30), conjugated to a carrier moiety (30, 31) and applied in CFA. However, when administered unconjugated in monomer form in IFA, these two peptide constructs failed to immunocastrate treated pigs. Dimerization of these constructs, as well as dimerization of the GnRH(C) monomer peptide or the GnRH analog Nafarelin via a carboxy-terminal cysteine, did not enhance effectivity of these antigens. Tandem-GnRH in absence of a carrier moiety also lost part of its effectivity when applied in lower dose or when CFA was replaced by IFA for the first vaccination, however, dimerization restored effectivity to a great extent. The importance of the tandem principle was confirmed by the observed selective potential for immunocastration of tandem and tandem-dimer constructs only, in contrast to the ineffectiveness of monomer and dimer constructs. Amino acid substitution of Gly-6 (G) by D-Lys-6 (k) did not hamper effectivity, however, not all amino acid substitutions are tolerated as shown by the negative results with the tandem-dimer formula of the GnRH-analog Nafarelin. Dimerization of three tandem formulas, viz. Tandem-GnRH with C-terminal or N-terminal cysteine or G6k-GnRH-tandem with C-terminal cysteine, rendered a higher effectivity to these unconjugated constructs, although no single formula was able to immunocastrate all pigs when administered as 1 mg unconjugated peptide in IFA. Therefore, it was deemed necessary to conjugate the antigen to a carrier molecule. We then investigated the possibility of resorting to milder adjuvants with similar efficaciousness and application of lower doses of the vaccine conjugate. By selectively replacing Gly-6 (G) from the original decapeptide sequence by D-Lys (k) a “handle” was created to covalently link the carrier protein ovalbumin to the peptide. A peptide dose of 500 µg (1.5 mg of conjugate) administered in the mild oil adjuvants Specol or double oil emulsion effectively immunocastrated pigs, and subsequently peptide dosis could even be lowered to 5-50 µg maintaining almost complete effectivity in reducing testis weight to less than 150 g and therefore presumably the occurrence of boar taint. Androstenone levels have not been determined in every experimental animal. However, when determined in animals having low testis weight after immunization androstenone levels never exceeded 0.5 µg/g, as

reported previously (26). In fact, most responders had androstenone levels below the sensitivity limit of the assay

Previously we have shown that immunocastration reduces testosterone to very low levels (26). As LH is necessary for testosterone production we determined LH levels to investigate if the expected downregulation of GnRH by immunization leads to low levels of LH, compared to untreated pigs and compared to the level before immunization. However, levels of LH fluctuate in time due to the pulsatile nature of pituitary LH secretion. To obtain detailed information on LH secretion it would have been necessary to take multiple frequent blood samples to get individual LH-profiles. A single blood sample for LH will not give complete information on the fertility status of an animal. It will reflect the balance of pituitary LH secretion stimulated by GnRH and the downregulation of the increases in LH by LH-stimulated testosterone levels in testicular intact animals, as can be seen from the wide range of values in the different groups. Despite these objections, we clearly see a significant reduction in median pLH levels after successful immunocastration, most probably reflecting diminished LH production by lack of GnRH. This next leads to reduced testosterone production by lack of LH (26). The much higher median pLH levels seen in barrows most probably reflect the lack of negative feedback by testicular steroids on pituitary LH secretion and represent the well-known increase in pituitary gonadotropin secretion seen after castration or gonad dysfunction.

Boar taint will develop in a significant percentage of sexually mature entire male pigs (boars), because boar testes produce the sex steroid and pheromone androstenone (6, 7). Androstenone is stored mainly in fat tissue (1-4) and salivary glands (8), and boar odor is mainly perceived upon heating the meat. However, the male steroid hormone testosterone, also produced in the testes, is involved in the better growth, more efficient feed conversion and higher carcass quality of boars. Pigs that have been castrated at very young age have thus been deprived of their source of testosterone entirely. Compared to boars castrated pigs (barrows) convert feed poorly and meat/fat ratio is less, resulting in economic losses for farmers (10-12). Nevertheless, the risk of tainted carcasses is considered unacceptable. Therefore, in many European countries young male pigs used for meat production are castrated to prevent the occurrence of boar taint.

Effective immunization against GnRH offers an alternative method to prevent the occurrence of boar taint. Because the first immunization takes place at the age of 10 weeks, and the very effective second (booster)immunization at the age of 18 weeks, the pigs can profit from the presence of testosterone until the time that indeed an effective amount of anti-GnRH-antibodies has been produced. Anti-GnRH titers appeared to be maximal at 12 wpv, i.e. at an age of 22 weeks. The production of anti-GnRH-antibodies takes place amply in time to entirely inhibit the production of androstenone, responsible for boar taint, before the pig is being slaughtered (37, 38). We have not extensively evaluated the skatole

response after immunization. However, in our previous experiments (26) only five (three immunocastrates, one non-responder and one control pig) of the 59 animals tested for skatole had skatole levels exceeding 0.25 μg/g (sensory threshold; 18) and no correlation was seen with testis weight or with androstenone level. Using the above immunization schedule the extreme reduction in testis size present before slaughter could indicate that due to absence of testosterone production in the very small testes these pigs would be at best intermediate between barrows and boars with respect to performance characteristics. When male pigs were immunized against GnRH by priming the immune system at early age and postponing the booster immunization to two weeks before slaughter, androstenone levels were decreased by the immunization but the economic performance of the immunocastrated pigs was improved to a level not different from boars (24) . However, the immunized pigs showed only a marginal reduction in testis size, which means that the absence of androstenone and therefore the risk for boar taint cannot be readily evaluated by visual inspection but needs additional testing, e.g. androstenone determination in backfat. Nevertheless, immunocastration by neutralizing GnRH seems the best feasible option to circumvent surgical castration. After optimization of immunization schedules with respect to production characteristics and easy detection of properly immunocastrated pigs, this option would allow the farmer to profit from the improved performance characteristics of immunocastrates, i.e. boar-like feed efficiency and carcass quality, compared to barrows (15, 36).

In conclusion, the data from our experiments prove that immunocastration of pigs using an optimized vaccine comprising a modified GnRH-tandem-dimer-ovalbumin conjugate administered in a mild adjuvant, G6k-GnRH-tandem-dimer-OVA in Specol, not only is very effective but also makes it possible to effectively counter the disadvantages of earlier immunocastration methods. The improved vaccine is highly efficacious, cost-efficient and marketwise acceptable, thereby providing a practically feasible alternative for surgical castration in pigs.

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Chapter 4

The role of the individual amino acids of a GnRH-tandem-dimer peptide used as an antigen for immunocastration of male piglets determined with systematic alanine replacements

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ABSTRACT
Immunocastration targetting gonadotropin releasing hormone (GnRH) can be obtained in male piglets using native GnRH conjugates. However, due to insufficient efficacy of these conjugates, improved GnRH antigens, like peptides existing of repeats of the GnRH amino acid sequence, have been designed. We previously reported about a dimerised GnRH-tandem peptide with a D-Lys at position 6 of the native GnRH sequence (G6k-TD) being highly effective. To evaluate the contribution of each individual amino acid of the GnRH decapeptide to the efficacy of the G6k-TD peptide, each amino acid was replaced consecutively by alanine (Ala-scan). The G6k-TD peptides were conjugated to ovalbumin, used for immunisation and tested for their ability to elicit GnRH antibodies and to immunocastrate male piglets. The results show that 4 out of 9 amino acids (pGlu-1, Ser-4, Arg-8 and Gly-10) can be replaced by alanine without negatively affecting immunocastration efficacy. Replacement of amino acids in other positions (Tyr-5, Leu-7 and Pro-9) gave partial decrease of efficacy: respectively 5, 6 and 6 out of 7 piglets were immunocastrated. Replacements at two other positions (His-2 and Trp-3) completely negated immunocastration activity. Thus, 7 out of 9 amino acid positions in the basic unit of G6k-TD can be substituted by alanine without affecting immunocastration efficacy.

1. INTRODUCTION

Immunisation against gonadotropin releasing hormone (GnRH) is an proven method for blocking the hypothalamus-pituitary-gonadal axis. In most studies the antigen consisted of a decapeptide, identical to native GnRH, chemically conjugated to a carrier protein. In general more than two vaccinations, a high peptide dose or a strong adjuvant were needed, to raise antibodies that were able to affect the hypothalamus-pituitary-gonadal axis [1-8]. This is probably necessary to counteract the insufficient immunogenicity of the GnRH peptides used [9-10]

We showed that immunogenicity can be improved by using a GnRH-tandem molecule [11-12]. Modulation based on these GnRH-tandem molecules produced further improvements. By replacing the glycine residue at positions 6 and 16 of the GnRH-tandem molecule by D-Lys an efficient immunocastration antigen was designed [13]. Because no relevant data are available with respect to the role of the individual amino acid residues with respect to immunocastration efficacy, we replaced each amino acid one at a time by alanine and tested these constructs for their ability to immunocastrate male piglets (Ala-scan).

2. MATERIALS AND METHODS

2.1 Materials

Acetonitrile (ACN) was HPLC-S gradient grade, N-methylpyrrolidone (NMP), diisopropylethylamine (DIEA), dimethylformamide (DMF), trifluoroacetic acid (TFA) and piperidine were peptide synthesis grade and were all obtained from Biosolve (Valkenswaard, NL). N-hydroxybenzotriazole (HOBt) and 2-(1H-benzotriazol-1-yl-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) were obtained from Richelieu Biotechnologies Inc. (Hamon, Canada). Benzotriazol-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP) was obtained from Novabiochem (Laufelfingen, Switserland). Thioanisole (TA), ethanedithiol (EDT), dimethylsulfoxide (DMSO), pentane and dimethylaminopyridine (DMAP) were pro-analysis grade and were obtained from Merck (Darmstad, Germany). Diethylether was purified over a column of activated basic aluminumoxide before use. Amino acid derivatives and resins were obtained from Bachem Feinchemicalien AG (Bubendorf, Switzerland).

2.2 Multiple peptide synthesis (MPS)

A Hamilton Microlab 2200 was programmed to deliver washing solvents and reagents to a rack with 40 individual 4 ml columns with filter, containing 30 mmol of resin for peptide synthesis. The columns were drained after each step by vacuum. The coupling cycle was based on Fmoc chemistry using double coupling steps and HBTU as activation reagent [14].

After coupling of the last amino acid, the Fmoc group was cleaved with 30% piperidine/NMP, the peptides were washed, acetylated in 30 min using NMP/aceticanhydride/DIEA 10/1/0.2, washed again, and dried. The peptides were deprotected and cleaved in 2 hr in a mixture of 1.5 ml of TFA/phenol/TA/water/EDT 10/0.75/0.5/0.5/0.25. The cleavage mixture was filtered, the resin was washed with 0.5 ml TFA, and the peptide was precipitated by adding 13 ml of pentane/diethylether 1/1. After centrifugation, the precipitate was extracted again with pentane/diethylether. The precipitate was dried, dissolved in ACN/water 1/1 and lyophilized. This procedure yields, depending on molecular weight, 25 to 70 mg of peptide.
Peptide sequences synthesized are summarized in table 1 in single letter code.

2.3 Analytical HPLC

For analysis of peptides, we used a LC-MS (electrospray) system, which consists of two Waters pumps model 510, a Waters gradient controller model 680, a Waters WISP 712 autoinjector, and a Waters 991 photodiode array detector. The mass spectrometer was a Micromass Quattro II sq, which was used in positive ion mode. Products were analyzed in a linear gradient from 10% ACN/water with 0.05% TFA to 70% ACN/water with 0.05% TFA in 30 min on a Waters Delta Pak C18-100Å (3.9x150 mm, 5 mm) column at 1 ml/min at 215 nm.

2.4 Dimerisation

Crude products were dimerised via the SH-group of the C-terminal cysteine, by dissolving the products in 20% DMSO in water and adjusting the pH to 5 - 6 with 1% NH_4HCO_3. The final concentration was 40 mg/ml. After stirring at room temperature for at least 5 h, the dimerisation process was monitored by analytic HPLC as described above. After dimerisation was completed for more than 80% the products were stored at –20 °C until purification.

2.5 Preparative HPLC

Peptides purifications were carried out using a Waters Prep 4000 liquid chromatograph, equipped with a Waters RCM module with two PrepPak cartridges plus guard cardridge (40x210 mm or 25x210 mm) filled with delta-Pak C18-100Å (15 mm) material. In general, purifications were run using the same eluents as in analytical HPLC, but at a gradient speed of 0.5% ACN/ min and a flow rate of 40 or 100 ml/ min. Peptides were detected at 215-230 nm using a Waters 486 spectrophotometer with a preparative cell. The peptides were lyophilized and purity was determined using analytical HPLC.

2.6 Conjugate preparation

For conjugation via N-ethyl-N-(3'-dimethylaminopropyl)carbodiimide hydrochloride (EDC) to ovalbumin (OVA) an equal weight of both the peptide and the carrier protein were dissolved separately in milli Q-water and both solutions were mixed well. Next a 10-fold excess, based on weight equivalents, of EDC was dissolved in milli Q-water. Subsequently, this solution was slowly added to the solution of peptide/OVA under continuous stirring, pH of this final solution was 5. After at least 6 h slowly shaking the product was dialyzed (MW cut-off 10,000) against a 300 times excess of milli Q-water for two days. Water was refreshed twice a day. The loading was calculated from comparative amino acid analysis of the conjugate and the carrier protein. Amino acid analysis was performed using a Waters Pico-Tag system, after hydrolysis in a Pico-Tag workstation using 6N HCl at 150 °C for 1 hour, and derivatization with phenylisothiocyanate.
The G6k-GnRH tandem dimer OVA conjugate is abbreviated by G6k-TD. The conjugates with alanine substitutions are abbreviated by the amino acid in the native GnRH sequence that is replaced, its position and an A for the alanine replacement, for instance pE1A-G6k-GnRH tandem dimer OVA conjugate is abbreviated by pE1A.

2.7 Emulsion preparation

Specol consisting of two detergents in a light mineral oil was used as oil phase [15]. The water-in-oil-(WIO)-emulsions were prepared using an Ultra Turrax. Specol (5 parts v/v) was brought into a 25 ml glass vessel and the waterphase (4 parts v/v) consisting of the conjugate in milli Q water was slowly added while the emulsion was stirred. After the waterphase was added, the emulsion was stirred for half a minute at the same rotation speed (15000 rpm). Emulsions were stored overnight at 4°C to check stability and were administered to the animals the next day.

2.8 Animals

Seventy four male piglets, approximately 10 weeks of age, were involved in this experiment. The crossbred piglets were housed in half slatted pens at ID-Lelystad experimental farm Bantham. The pigs were kept according to the current practice and were given *ad libitum* access to feed and water.

2.9 Immunisation

The piglets were randomly assigned to the eleven treatments. All animals were injected with 2 ml emulsion containing the dimerised tandem GnRH conjugates (i.e. 62 µg peptide) or an emulsion without antigen. Injections were administered intramuscularly in the neck at the start of the experiment (day 0) and 7 weeks later (7wpv). Thirteen weeks after initial immunisation (13 wpv) the animals were slaughtered.

2.10 Measurements and blood sampling

Animals were weighed at day 0 and at 7 and 13 wpv. Testis sizes were determined by measuring testis length with a vernier calipers at day 0, and 7, 10 and 13 weeks thereafter. Testis sizes were recorded as average of both testes.

Bloodsamples were taken via puncture of the vena jugularis on the same days testis sizes were measured, and also 4 weeks after the initial immunisation. Blood samples were kept overnight at 4□C and the next day serum was obtained by centrifugation (1500 g, 15 min). Serum samples were stored at -20°C until assayed.

2.11 Evaluation after slaughter

After slaughter testes were removed, dissected free of epididymides and weighed. Testes weights were recorded as average of both testes.

2.12 Peptide antibodies

Antibodies to the peptides used for immunisation were determined with an ELISA. Peptides were coated in the wells of a microtitreplate using glutardialdehyde (GDA). GDA was coated to the surface of the wells by incubation with 0.2% GDA in 0.1 M phosphate buffer (pH5) for 4 hours at room temperature. Plates were rinsed 3 times for 10 minutes with 0.1 M phosphate buffer (pH 8). One microgram of peptide in 100 µl phosphate buffer (0.1 M, pH 8) was coated per well by incubating for 3 hours at 37°C. Plates were stored at -20°C until used. Thawed plates were rinsed 3 times for 10 minutes with milli-Q water containing 8.2 g NaCl, 1.15 g $Na_2HPO_4.2H_2O$, 0.20 g $NaH_2PO_4.2H_2O$ and 5 ml of a 10 %Tween 80 solution in water per litre water.

Serial serum dilutions of the antipeptide sera were allowed to react with the coated peptides for 1 hour at 25°C. After rinsing for 3 times 10 minutes goat-anti-pig IgG coupled to horseradish peroxidase (Dako, Glastrup, Denmark) was introduced as second antibody for 1 hour and ABTS (Boehringer, Mannheim, Germany), 250 µl (2 g/100 ml) in 10 ml substrate buffer to which 20 µl H_2O_2 (3% solution) was added, was used as substrate. Absorption was measured at 405 nm. The titer was calculated as the - log of the dilution factor that gives an optical density 4 times background.

2.13 GnRH antibodies and testosterone

Antibodies to GnRH were determined as described previously [11]. Serial dilutions of the pig antisera were allowed to bind to ^{125}I-GnRH. GnRH binding capacity is expressed as percentage binding of ^{125}I-GnRH at a given serum dilution.

Testosterone levels in serum were determined using a Coat-a-Count kit purchased from DPC laboratories, Los Angeles, CA.

3. RESULTS

3.1 Peptides and conjugation

Crude peptides were 40-70% pure according to the peak area as determined by analytical HPLC at 215 nm. After dimerisation the peptides were purified using preparative HPLC. The yield was 10 - 50% and purity was determined to be at least 90%.
Alanine replacement did affect solubility especially of the P9A-G6k-GnRH tandem peptide. Solubility of this peptide was low and it had to be diluted further for the dimerisation procedure. Due to the low solubility of this product, the conjugation efficacy was low. According to amino acid analysis this conjugate (P9A) contained 0.16 mg peptide per mg ovalbumin. Conjugation efficacy varied between 0.30 and 0.50 mg peptide per mg carrier protein for the remaining conjugates.

Table 1. Amino acid sequences of peptides

peptide	amino acid sequence
G6k-GnRH-tandem:	pEHWSYkLRPGQHWSYkLRPGC#
pE1A-G6k-GnRH-tandem	*A---------A----------#
H2A-G6k-GnRH-tandem	-A---------A---------#
W3A-G6k-GnRH-tandem	--A---------A--------#
S4A-G6k-GnRH-tandem	---A---------A-------#
Y5A-G6k-GnRH-tandem	----A---------A------#
L7A-G6k-GnRH-tandem	------A---------A----#
R8A-G6k-GnRH-tandem	-------A---------A---#
P9A-G6k-GnRH-tandem	--------A---------A--#
G10A-G6k-GnRH-tandem	---------A---------A-#

pE = pyroglutamic acid; * = acetyl; # = amide; k = D-lysine; G6k = G at position 6 in the native GnRH sequence substituted by k; - = amino acid at this position does not differ from the amino acid at the same position in G6k-GnRH-tandem; The D-Lys at positions 6 and 16 was not involved in this Ala-scan, as it was used for conjugation purposes.

3.2 Testis size and testis weight

Seven weeks after the first immunisation, initial signs of immunocastration could be observed for three vaccine groups, R8A, G10A and G6k-TD (see table 1 for peptide identification). Pigs of these groups showed hardly any increment of average testis size at the time of booster immunisation (data not shown). These preparations were found to be 100% successful with respect to immunocastration. Other vaccine groups that were fully effective are pE1A and S4A, while in the Y5A, L7A and P9A groups respectively 2, 1 and

1 animals did not respond to the immunisation (see figure 1). Animals vaccinated with H2A and W3A were not immunocastrated at all, with testes weighing more than 131 grams. Individual testis weight of immunocastrated animals did not exceed 70 grams, resulting in a clear difference between immunocastrated and not immunocastrated animals.

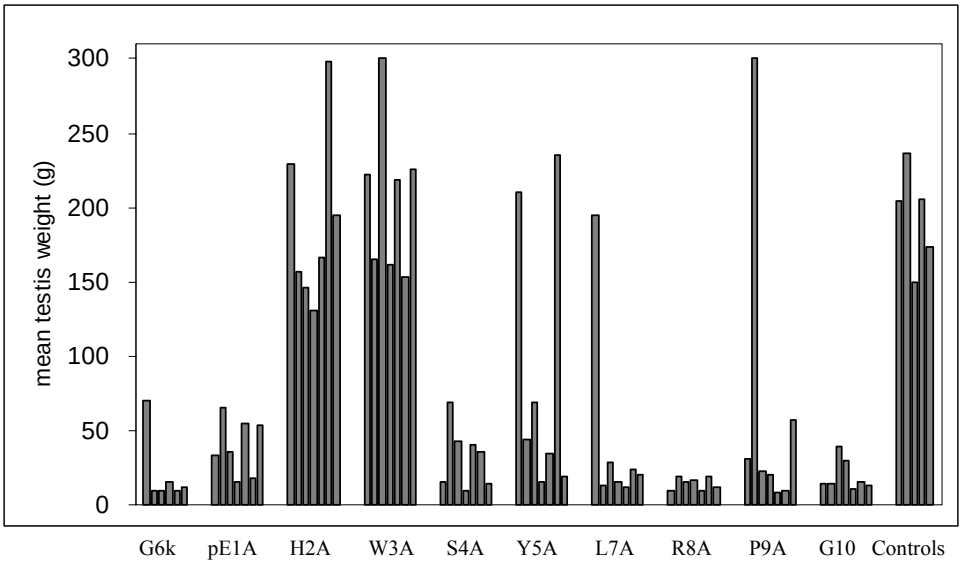

Figure 1.Mean testis weight per individual pig after immunisation with G6k-GnRH-tandem-dimer peptide conjugated to ovalbumin or with tandem-dimer peptides with amino acids replaced by alanine conjugated to ovalbumin.

3.3 Antibody response

Mean antibody titers against the peptides used for immunisation are given in table 2. Antibody titers of individual animals against the different peptides ranged from 2 to 4. Within the vaccine groups Y5A, L7A and P9A the not-immunocastrated animals showed lower anti-peptide titers as compared to the immunocastrated animals. Animals vaccinated with H2A and W3A were not immunocastrated, although significant antipeptide titers were present. However, GnRH binding percentages at 1/ 2000 serum dilution were undetectable or low in H2A and W3A groups. Only at a 1/200 serum dilution a low binding percentage was measured in sera of all animals of group H2A and three animals of group W3A (data

not shown). Thus, animals with low GnRH binding percentages were not immunocastrated. High GnRH antibody levels did always result in successful castrated animals.
Testis weight of animals with intermediate GnRH antibody levels varied from 15 to 300 gram. The relation between testis weight and average GnRH binding percentage per animal during the experiment is shown in figure 2.

Table 2. Effect of different vaccines on testis weight, number of responders, peptide antibody titer, GnRH binding percentage and testosterone levels in serum[a].

Vaccine	Testis weight (g), median (range)	Number responders/ total number	Anti-peptide titer (mean)	GnRH binding (mean)	Testosterone (pmol/ml) at 13 wpv[b] median (range)
pE1A	36 (16-65)	7/7	3.24	15.7	<0.3 (<0.3-3.80)
H2A	166 (131-298)	0/7	2.69	2.0	6.80 (2.06-24.32)
W3A	218 (153-300)	0/7	3.39	1.3	4.75 (0.46-11.72)
S4A	36 (10-69)	7/7	3.19	16.9	<0.3 (<0.3)
Y5A	44 (15-235)	5/7	2.92	11.3	<0.3 (<0.3-9.44)
L7A	20 (12-195)	6/7	3.52	17.6	<0.3 (<0.3-48.91)
R8A	15 (10-19)	7/7	3.34	17.7	<0.3 (<0.3)
P9A	23 (8-300)	6/7	2.62	15.8	<0.3 (<0.3-11.57)
G10A	14 (11-39)	7/7	3.19	17.6	<0.3 (<0.3)
G6k-TD	11 (10-70)	6/6	3.32	17.2	<0.3 (<0.3-1.18)
Controls	204 (150-236)	0/5	n.d.[c]	n.d.	2.05 (0.65-9.35)

[a] A responder is defined as a pig with substantially reduced testis weight as compared to control pigs. In this study testis weight of responders, non-responders and control animals, ranged from 8-70 g, 131-300 g and 150-236 g respectively. GnRH binding is given as the mean binding percentage of the 4 bleeds (5, 7, 10 and 13 wpv) per animal at a 1/2000 serum dilution. These values per animal are averaged for all animals of a treatment. The anti-peptide titer was calculated as the log of the dilution factor that gives an optical density four times background of 10 wpv antiserum. [b] weeks post vaccination, [c] not detectable.

3.4 Serum testosterone levels

Testosterone levels in all immunocastrated animals were undetectable or low at the time of the second immunisation, and decreased further thereafter (results not shown). A majority (n=31) of these animals reflected a castration effect as early as five weeks after initial immunisation as measured by undetectable serum testosterone levels at that time. Testosterone levels were also undetectable at the time of booster immunisation. Testis

weight of these animals varied from 8-36 grams. Final testosterone levels are shown in table 2.

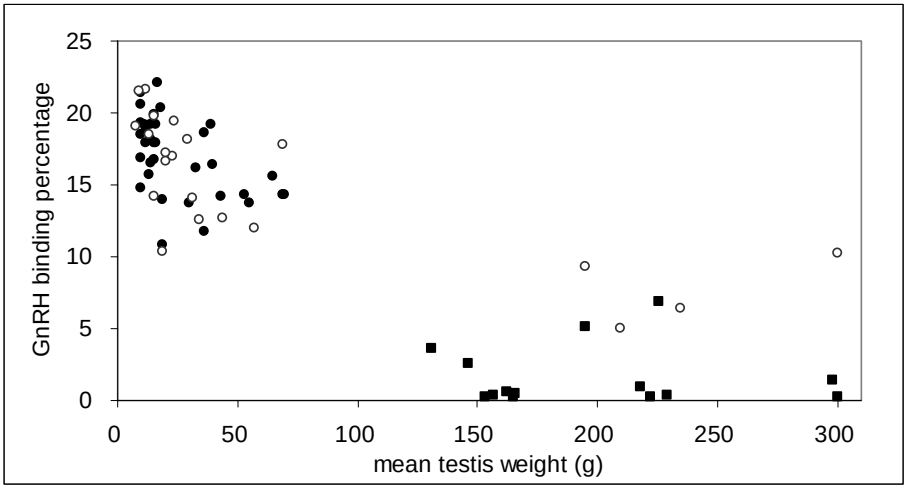

Figure 2.Correlation between the mean GnRH binding percentage per animal (mean GnRH binding percentage of 5, 7, 10 and 13 wpv serum at a 1/2000 dilution) and mean testis weight per animal of pigs of fully effective (•), partially effective (O) and not effective treatments (■).

4. DISCUSSION

In a previous study we have shown that dimerisation of D-Lys^6 GnRH tandem results in a highly immunogenic GnRH vaccine which induces immunocastration in young male piglets [13]. In order to determine the role of the individual amino acids of this GnRH antigen necessary for induction of effective GnRH immunocastration, an in vivo Ala-scan was performed. The amino acids of the native decapeptide D-Lys^6 GnRH were replaced one by one by alanine. The D-Lys at positions 6 and 16 was not involved in this Ala-scan, as it was used for conjugation purposes. After dimerisation of the tandem peptides, conjugation was performed and antigens were tested in young male pigs.

The results show that 4 out of 9 amino acids (pGlu-1, Ser-4, Arg-8 and Gly-10) can be individually replaced by alanine without affecting immunocastration efficacy. Replacement of amino acids in three positions (Tyr-5, Leu-7 and Pro-9) gave partial decrease of efficacy, as 5 or 6 out of 7 piglets were immunocastrated, while replacements at two other positions (His-2 and Trp-3) completely negated immunocastration activity (Table 3).

Table 3. Replaceability of individual amino acids by A in each decapeptide of the G6k-GnRH tandem dimer with respect to immunocastration efficacy.

	pE	H	W	S	Y	k	L	R	P	G
Replaceable by A	++	- -	- -	++	+	n.a.	+	++	+	++

n.a. not applicable, - - not replaceable, + partially replaceable, ++ replaceable.

The relation between GnRH binding antibodies, serum testosterone and testis weight, was as expected. Sera with a high GnRH binding capacity, showed low serum testosterone levels and correlated with small testes. Testicular weights of immunocastrated animals were less than 70 grams. Animals that were not castrated had testes weighing more than 131 grams. In previous studies we assumed testis weight of successfully immunocastrated animals to be less than 150 grams [12]. However, in the current experiment some animals that were not immunocastrated showed testis weights less than 150 gram, due to the younger age of the pigs at slaughter.

The Ala-scan reported in this paper revealed properties of the individual amino acid residues that are important for inducing immunocastration. It is demonstrated that vaccination with peptides with alanine substitions at position His-2 or Trp-3 are not able to establish immunocastration in male piglets. Antibodies raised against these peptide conjugates hardly bound to GnRH, indicating that only a very small part of these antibodies recognizes GnRH.

Immunisation with Y5A, L7A and P9A was partially effective, with respectively 5, 6 and 6 animals out of 7 being immunocastrated. Antipeptide titers as well as GnRH binding in the RIA did correlate with immunocastration efficacy for these groups. This indicates that antibodies raised against Y5A, L7A and P9A bind native GnRH, however, the amount of antibodies is not sufficient to establish immunocastration in all animals.

pE1A, S4A, R8A and G10A were fully effective in immunocastrating male piglets. Individual testis weights per group suggest that R8A and G10A are the best antigens as compared to G6k-TD.

On average binding of GnRH by antibodies correlates with testis weight, however, GnRH binding percentages are not prognostic for testis weight of individual animals. High and low titered sera resulted in small and heavy testis respectively, while testis weight of pigs with intermediate GnRH antibody levels varied from 15 to 300 gram (figure 2).

We found that replacements of positions 2 and 3 by alanine did not result in immunocastration, as these antigens did not raise specific GnRH binding antibodies. In contrast, we reported previously [13] that a dimerised GnRH peptide in which both pGlu-1 and His-2 were replaced by respectively cysteine and lysine [16] was effective in immunocastration. However, this peptide did not contain a D-lysine at position 6, it was not

in the tandem form and it was dimerised and conjugated via the N-terminus. Apparently in such a construct the impact of substitution of the N-terminal amino acid residues on the efficacy of the vaccine is not as great as in our C-terminally dimerised tandem construct. Further, immunisation with GnRH peptides with N-terminal amino acid deletions, including His-2 and Trp-3, i.e. GnRH 4-10 [17] or His-2 alone, i.e. GnRH 3-10 [18] also appeared to be effective in inducing GnRH neutralising antibodies and immunocastration. These findings strongly suggests that the results of the present study may not be generally applicable.

Substitutions with other amino acids than alanine where also tested in a previous study [13], but only for position 6 of the G6k-GnRH-tandem dimer in a unconjugated form. It appeared that substitution of D-Lys 6 by glycine did not hamper effectivity, whereas substitution by 2-naphtyl-D-alanine (in similarity to the GnRH analog Naferalin), was not tolerated. In general, it is expected that immunocastration efficacy after amino acid replacement depends on similarity of the physical or chemical properties of the particular amino acids.

This study shows that an *in vivo* alanine-scan of an antigen to be used for immunisation, is an effective method to define the essential amino acid residues in antigens. We conclude that 4 (pGlu-1, Ser-4, Arg-8 and Gly-10) out of 9 amino acids residues of the G6k-TD peptide can be replaced by alanine without losing the ability to immunocastrate male piglets. Three (Tyr-5, Leu-7 and Pro-9) out of 9 amino acids are more important, while two others (His-2 and Trp-3) are essential with respect to the efficacy of these peptides to induce immunocastration. Thus, for the purpose of an immunocastration vaccine only two positions in the basic unit of G6k-TD do not allow substituted by alanine, indicating that effective immunocastration can be achieved with modified GnRH peptides.

5. ACKNOWLEDGEMENTS

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Chapter 5

GnRH-tandem-dimer peptides for inducing an immunogenic response to GnRH-I without cross-reactivity to other GnRH isoforms

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In press

ABSTRACT
Gonadotropin-releasing hormone (GnRH) occurs in various isoforms in mammals, i.e. GnRH-I (mammalian GnRH), GnRH-II (chicken GnRH-II), GnRH-III (salmon GnRH) and two forms of lamprey GnRH. The functions of the latter four molecules have only been partially investigated. Also not much is known about the physiological effects of GnRH-I immunization on the function of these GnRH isoforms. In order to avoid possible harmful side effects due to undesired neutralization of GnRH isoforms, GnRH-I specificity of antibodies raised against a panel of alternative GnRH antigens was determined. The results show that GnRH antigens can be designed which generate antibodies that specifically bind GnRH-I, without cross-reacting with other GnRH isoforms.

1. INTRODUCTION

In mammals the decapeptide gonadotropin releasing hormone (GnRH-I) triggers the synthesis and release of luteinising hormone (LH) and follicle stimulation hormone (FSH) from the pituitary. LH and FSH are responsible for steroidogenesis and gametogenesis. Recently, additional isoforms of GnRH were identified in mammals. GnRH-II, a molecule with an amino acid sequence similar to chicken GnRH-II was cloned from a human genomic library [1]. A third GnRH isoform (GnRH-III) with a similar amino acid sequence as salmon GnRH, was extracted from the bovine and human brain [2]. Immunohistologically, the presence of GnRH isoforms (lamprey GnRH-I, lamprey GnRH-III) that were first detected in lamprey was demonstrated in the mammalian brain [3, 4]. The function of the various isoforms in mammals, however, is ill defined. Most likely they play a role in fertility regulation. GnRH-II and GnRH-III are able to induce the secretion of gonadotropines [2, 5-8]. The presence of GnRH-II in the human endometrium and the fact that GnRH-II is released from the early human placenta indicates that it may be involved in embryo implantation and regulation of pregnancy [9, 10]. It has also been suggested that the GnRH isoforms are involved in sexual behaviour [2, 11, 12]. In contrast to GnRH-II and GnRH-III, lamprey GnRH-III does not induce LH secretion. This isoform seemed to selectively activate FSH release, indicating that it could be the FSH-releasing factor [13-15]. However, this could not be confirmed by others [16].

GnRH-II is not only expressed in the brain, but also in tissues outside the brain. High concentrations were determined in the kidneys, bone marrow and prostate [1]. This indicates that GnRH-II may also have functions unrelated to fertility regulation. Indeed, neutralization of GnRH-II by active immunization, led to a slight increase in kidney weight suggesting a role for GnRH-II in kidney function [17].

Immunization against GnRH-I is shown to be an efficient method to block fertility and reproduction in male and female mammals [18-21]. In addition, immunization suppresses hormone dependent malfunctions, such as breast cancer and prostate cancer [22-26]. However, with the new insight, that several GnRH isoforms are present in mammals, it is not known which of the GnRH isoforms are targetted by GnRH-I immunization. Although all GnRH isoforms seem to be involved in fertility, some may have additional functions unrelated to fertility. Possible undesired effects of GnRH-I neutralization on these unrelated functions must be circumvented. Therefore the specificity of antibodies raised against a panel of alternative GnRH antigens for the GnRH isoforms was studied. Here we report that some GnRH antigens elicite antibodies which cross-react with most of the GnRH isoforms, while others are only directed against GnRH-I, indicating that small spectrum isoform specific antibodies or wide spectrum isoform non-specific antibodies can be generated as desired.

2. MATERIALS AND METHODS

2.1 Antisera

Antisera were raised in young male pigs and directed against different GnRH antigens. Table 1 gives an overview of the peptides used for immunization, including amino acid sequence in single letter code.

Table 1. Amino acid sequences and abbreviations of peptides used for immunization[a]

GnRH antigen	Amino acid sequence	Abbreviation
GnRH-monomer	pEHWSYGLRPGC#	mono
GnRH-tandem	pEHWSYGLRPGQHWSYGLRPGC#	tandem
G6k-GnRH-tandem	pEHWSYkLRPGQHWSYkLRPGC#	G6k
pE1A-G6k-GnRH-tandem	*AHWSYkLRPGAHWSYkLRPGC#	pE1A
S4A-G6k-GnRH-tandem	pEHWAYkLRPGQHWAYkLRPGC#	S4A
R8A-G6k-GnRH-tandem	pEHWSYkLAPGQHWSYkLAPGC#	R8A
G10A-G6k-GnRH-tandem	pEHWSYkLRPAQHWSYkLRPAC#	G10A

[a] pE = pyroglutamic acid; * = acetyl; # = amide; k = D-lysine; G6k = Gly on position 6 in the native GnRH sequence substituted by D-lysine; Underlined amino acid differ from G6k-GnRH-tandem

Peptide synthesis, vaccine preparation and immunization procedure for mono and tandem have been decribed previously[13]. Briefly, the GnRH molecules synthesized with a C-terminal cysteine were conjugated to Keyhole Limpet Hemocyanin via maleinimidobenzoyl-hydoxysuccinimide ester. Conjugates were emulsified in Complete Freund Adjuvant and Incomplete Freund Adjuvant for the first and second immunization, respectively. The vaccines were administered to young male pigs at 10 and 18 weeks of age. Only sera from successfully treated pigs, as determined by undetectable serum testosterone levels and reduced testis weights were used in this study (3 and 5 pigs for the mono and the tandem group, respectively).

Synthesis of the G6k-GnRH-tandem peptides has been described previously [27]. The peptides were brought in the tandem dimer form by dimerization via the C-terminal cysteine, subsequent conjugation to ovalbumine was achieved using N-ethyl-N'-(3'-dimethyl-aminopropyl) carbodiimide. Antigens were administered with Specol adjuvant to young male pigs at 10 and 17 weeks of age. In the present study only sera raised against five fully effective G6k-GnRH-tandem dimer peptides are included [27]. Each group consisted of six (G6k) or seven (pE1A, S4A, R8A, G10A) pigs.

2.2 GnRH isoform peptides

The peptides used for the competitive radioimmunoassay were synthesized and analyzed as described previously [27]. Peptides were at least 90% pure. Amino acid sequences of the synthesized peptides are given in table 2 in a single letter code.

2.3 Competitive radioimmunoassay

Serum samples were diluted in PBS with 0.4% BSA (dilution buffer). Fifty µl serum dilution was put in microwell plates and pre-incubated with 25 µl GnRH isoform peptide solution. An unrelated peptide was included as negative control. This mixture was allowed to incubate for 24 hours at 4°C. The next day 25 µl iodinated GnRH-I (approximately 13000 cpm, Amersham Pharmacia Biotech, Buckinghamshire, England) was added to compete with the preincubated GnRH isoform peptides for binding to the antibodies. After incubation overnight (4°C) unbound peptide was separated from bound peptide using dextran coated charcoal. After centrifugation (2000 g, 15 min.) supernatant was separated, counted and the percentage iodinated GnRH bound to the antibodies was calculated.

3. RESULTS

In a competitive radioimmunoassay displacement of iodinated GnRH-I by the GnRH isoforms (table 2) was determined.

Competition between the iodinated GnRH-I and 250 nM GnRH-I, resulted in the expected reduction of binding of iodinated GnRH-I by the antisera for all sera. Iodinated GnRH-I was displaced for 70-100% by GnRH-I (mean value per group > 89%; figure 1a). Similar results were obtained with 25 nM GnRH-I (mean value per group > 88%), whereas 2.5 nM GnRH-I showed a lower reduction of displacement (mean value per group > 67%).

Table 2. Amino acid sequences of GnRH isoforms[a]

Peptide	Amino acid sequence
Mammalian GnRH (GnRH-I)	pEHWSYGLRPG#
Chicken GnRH-II (GnRH-II)	pEHWSHGWYPG#
Salmon GnRH (GnRH-III)	pEHWSYGWLPG#
Lamprey GnRH-I (lamprey GnRH-I)	pEHYSLEWKPG#
Lamprey GnRH-III (lamprey GnRH-III)	pEHWSHDWKPG#

[a] pE = pyroglutamic acid; # = amide; Underlined amino acid differ from GnRH-I amino acid sequence;

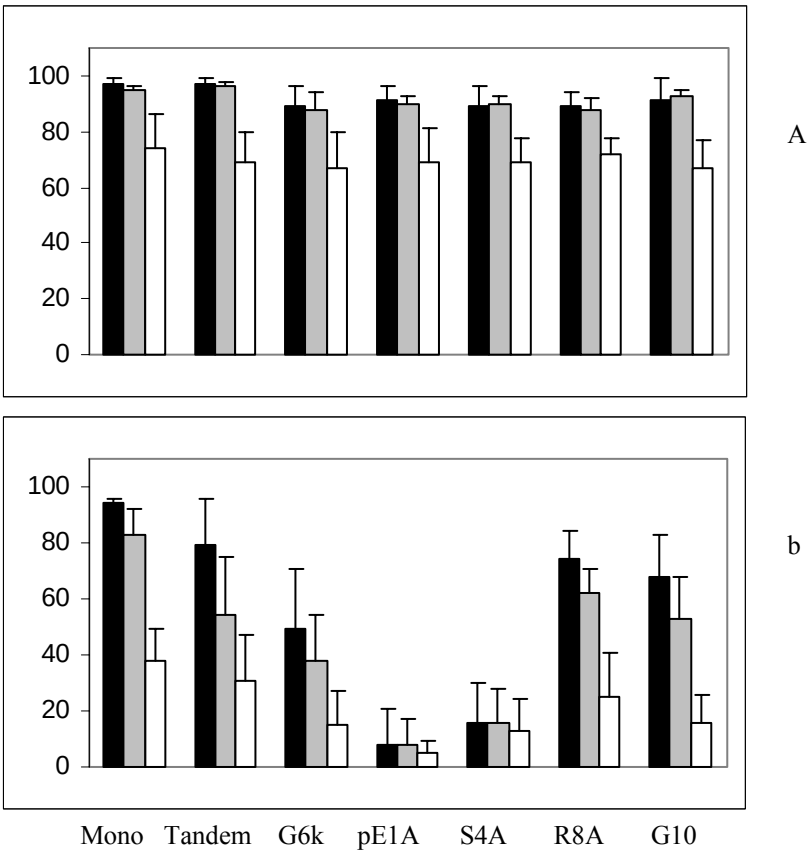

Figure 1: Percentage of iodinated GnRH-I displaced by GnRH-I (a) and GnRH-II (b) for sera raised against the different GnRH antigens as indicated on the x-axis and explained in table 1, respectively GnRH mono, GnRH tandem, G6k (GnRH-tandem-dimer peptide with a D-lysine on position 6 of the native decapeptide), pE1A, S4A, R8A and G10A (alanine replacement variants of G6k). Each bar represents the mean value of 3-7 sera (see materials and methods) raised against one antigen. The sera were obtained after the second immunization and diluted 1/10000. The concentrations of GnRH-I and GnRH-II for displacement were: 250 nM (black bars), 25nM (grey bars) and 2.5 nM (white bars).

In contrast, displacement of iodinated GnRH-I by 250 nM GnRH-II revealed clear differences between groups (figure 1b). Sera raised against pE1A and S4A, allowed hardly any displacement of iodinated GnRH-I by GnRH-II. Only one serum out of seven of both, the pE1A and the S4A vaccinated groups, showed a substantial displacement percentage

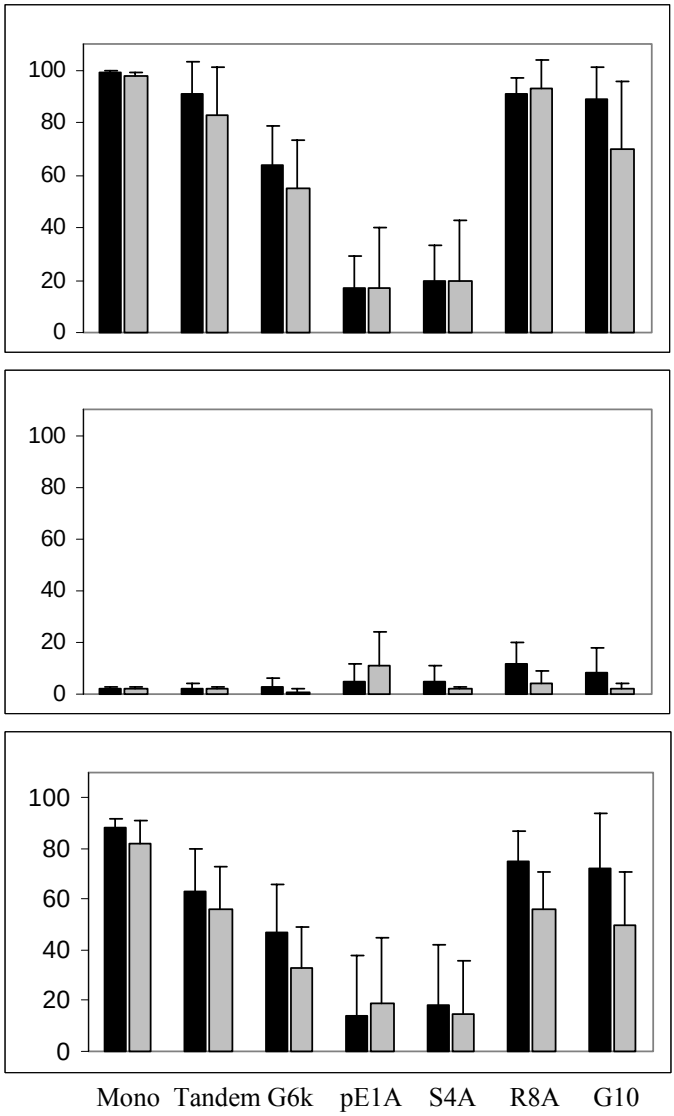

Figure 2: Percentage of iodinated GnRH-I displaced by GnRH-III (a), lamprey GnRH-I (b) and lamprey GnRH-III (c) for sera raised against the different GnRH antigens, respectively GnRH mono, GnRH tandem, G6k (GnRH-tandem-dimer peptide with a D-lysine on position 6 of the native decapeptide), pE1A, S4A, R8A and G10A (alanine replacement variants of G6k). Each bar represents the mean value of 3-7 sera (see materials and methods) raised against one antigen. The sera were obtained after the second immunization and diluted 1/10000. The concentrations of GnRH-III, lamprey GnRH-I and lamprey GnRH-III for displacement were 250 nM (black bars) and 25 nM (grey bars).

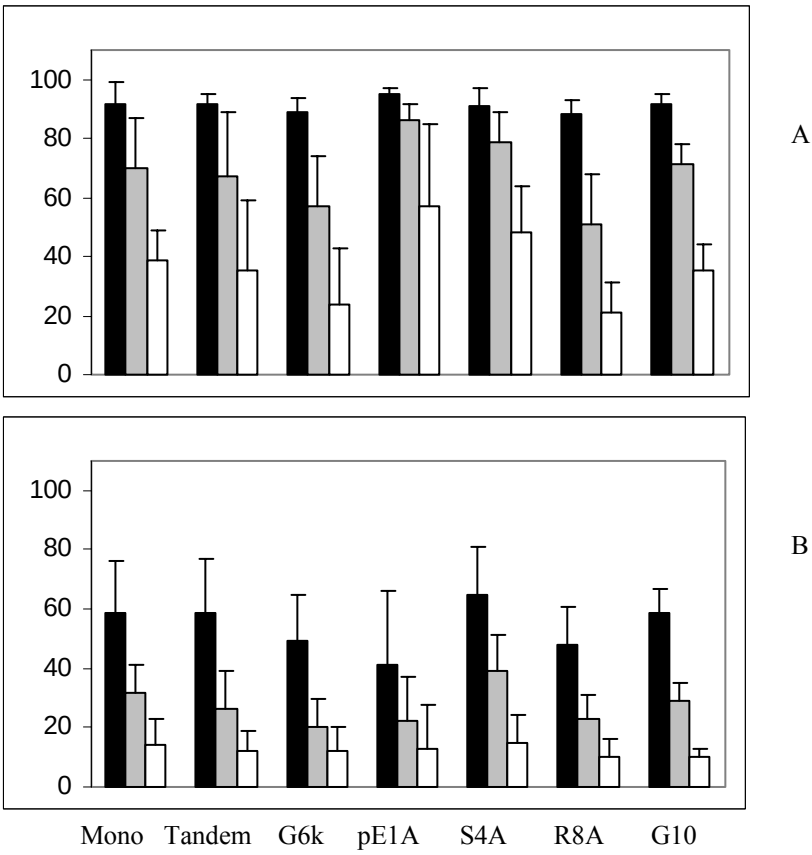

Figure 3: Percentage of iodinated GnRH-I displaced by GnRH-I (a) and GnRH-II (b) for sera raised against the different GnRH antigens, respectively GnRH mono, GnRH tandem, G6k (GnRH-tandem-dimer peptide with a D-lysine on position 6 of the native decapeptide), pE1A, S4A, R8A and G10A (alanine replacement variants of G6k). Each bar represents the mean value of 3-7 sera (see materials and methods) raised against one antigen. The sera were obtained at time of second immunization and diluted 1/1000. The concentrations of GnRH-I and GnRH-II for displacement were: 250 nM (black bars), 25nM (grey bars) and 2.5 nM (white bars).

(38% and 46% respectively), resulting in a mean displacement percentage of 8% and 16% for pE1A and S4A, respectively.

Sera raised against mono, tandem, G6k, R8A and G10A allowed greater displacement of iodinated GnRH-I by GnRH-II (mean displacement per group 94, 79, 49, 74 and 68% respectively). Competition with 25 nM and 2.5 nM GnRH-II showed a dose dependent

reduction of the displacement of iodinated GnRH for sera raised against mono, tandem, G6k, R8A and G10A. Subsequently displacement of iodinated GnRH-I by GnRH isoforms GnRH-III, lamprey GnRH-I and lamprey GnRH-III was investigated. Results are shown in figure 2. An unrelated peptide served as a negative control (data not shown). GnRH-III (250 nM) displaced iodinated GnRH-I in sera raised against mono, tandem, R8A and G10A, whereas 25 nM GnRH-III displaced similarly or slightly less as compared to the 250 nM dose. Lamprey GnRH-III displaced iodinated GnRH-I in a dose dependent manner, but displacement was lower as for GnRH-III. GnRH-III and lamprey GnRH-III displaced iodinated GnRH-I in sera raised against G6k, but to a lesser extent than for the previous mentioned groups. No displacement of iodinated GnRH-I by GnRH-III and lamprey GnRH-III was seen in sera raised against pE1A and S4A, except for low displacement percentages measured for one serum raised against pE1A and two sera raised against S4A. Lamprey GnRH-I and the control peptide were not able to displace iodinated GnRH-I.
To study the development of the specificity of the sera during the immunization period, displacement of iodinated GnRH-I by GnRH-I and GnRH-II was also determined for pre-booster sera collected at time of second vaccination. As expected iodinated GnRH-I was displaced by GnRH-I for pre-booster sera raised against all antigens (figure 3). However, in contrast to the observations for the post booster sera, 250 nM GnRH-II was able to displace iodinated GnRH-I in pre-booster sera raised against all antigens, including pE1A and S4A.

4. DISCUSSION

Recently, the presence of several GnRH isoforms in mammals has been demonstrated [1-4]. However, the functions of these GnRH isoforms in mammals have only partially been investigated. Most likely they all play a role in fertility regulation, but this role seems limited, as demonstrated in a mouse model. Mice which lack the GnRH-I gene do have GnRH-II producing cells as in normal mice but this is not sufficient to cause normal gonadal development [11]. The limited role of GnRH-II in fertility regulation is also shown in male rats [17]. Immunization against GnRH-II showed a slight but significant decrease of the seminiferous tubules diameter. However, GnRH-II may have functions, which are not related to reproduction, as is suggested by an increase in kidney weight of rats immunized against GnRH-II [17]. Indeed, high concentrations of GnRH-II were found in the kidneys and also in bone marrow and prostate [1]. Moreover, the widespread expression of GnRH-II receptor mRNA may also suggest functions of GnRH-II which are not related to reproduction [28], making it even more desirable to direct the antigenic response of a GnRH vaccine specifically towards GnRH-I.
Immunization against GnRH-I in order to block GnRH-I induced gonadal steroid secretion, may induce antibodies that cross-react with other GnRH isoforms, which may result in

undesired side effects because of unknown functions of the GnRH isoforms. In this study we evaluated the specificity of antibodies elicited by various GnRH antigens tested against different GnRH isoforms. The results show that GnRH-I specific antisera can be generated with dimerized G6k-GnRH-tandem peptides. Specificity is achieved by replacing a single amino acid in the decapeptide sequence of the antigen. Substitution of pyroglutamine at position one or serine at position four by alanine resulted in an immunogenic antigen, which induced antibodies that neutralize GnRH-I and blocked testes growth in male piglets, but did not cross-react with GnRH-II, GnRH-III or lamprey GnRH-III. We also conclude that the specificity of these antibodies is established after the second immunization, as antisera obtained at time of second immunization did not discriminate between the GnRH isoforms.

The antisera raised against pE1A and S4A allow displacement only with GnRH-I, while antisera raised against other antigens, mono, tandem, G6k, R8A and G10A allow displacement by GnRH-I, GnRH-II, GnRH-III and lamprey GnRH-III. We suggest that this can be explained by the position of the amino acid substitutions in the antigen (table 1). The results might indicate that two types of antibodies are generated, which define whether or not cross-reaction of the antisera with the GnRH isoforms occurs. One type of antibody binds the N-terminal part and the other type binds the C-terminal part of the GnRH antigens. The antigens of which the N-terminal part does not contain any substitutions (i.e. mono, tandem, G6k, R8A and G10A, table 3) generate antibodies against the N-terminus, which are able to bind GnRH isoforms without substitutions in the N-terminal part of the peptide (i.e. GnRH-I, GnRH-II, GnRH-III and lamprey GnRH-III).

Table 3. Amino acid alterations in GnRH antigens and GnRH isoforms as compared to native GnRH[a].

GnRH antigens		GnRH isoforms	
Mono	----------	GnRH-I	----------
Tandem	----------	GnRH-II	----▮-▮▮--
G6k	-----▮----	GnRH-III	------▮▮--
pE1A	▮----▮----	Lamprey GnRH-I	--▮-▮▮▮▮--
S4A	---▮-▮----	Lamprey GnRH-III	----▮▮▮▮--
R8A	-----▮-▮--		
G10A	-----▮---▮		

[a]Positions with amino acid replacements as compared with the native GnRH amino acid sequence are indicated with ▮

GnRH antigens with a substitution in the N-terminal part, i.e. pE1A and S4A, elicite antibodies against the N-terminus, which are not able to bind the N-terminal part of the GnRH isoforms. Antibodies raised against the C-terminus of pE1A and S4A only bind GnRH-I, resulting in GnRH-I specific antibodies. Lamprey GnRH-I did not bind either type of antibody as it has an amino acid substitution in both N- and C-terminal part of the peptide.
Antisera raised against mono, tandem, G6k, R8A and G10A showed displacement of iodinated GnRH-I by GnRH-I, GnRH-II, GnRH-III and lamprey GnRH-III in a competitive radioimmunoassay. However, differences in displacement were seen. GnRH-I showed the highest displacement activity, followed by GnRH-III, whereas lower displacement was shown for GnRH-II and lamprey GnRH-III. This order of displacement activity of the GnRH isoforms correlates with the number of amino acid substitutions in the C-terminal part of these peptides: with 0, 2, 3 and 4 amino acid substitutions for GnRH-I, GnRH-III, GnRH-II and lamprey GnRH-III respectively.
All sera raised against the mono allowed displacement by GnRH-I, GnRH-II, GnRH-III and lamprey GnRH-III. These results are in contrast to those of Ferro et al. [17], who did not find any cross-reactivity of antibodies raised against a GnRH-I analogue with GnRH-II. This discrepancy may be explained by the fact that in their GnRH analogue the N-terminal pyroglutamine was substituted by a cysteine for conjugation purposes. This is in accordance with our results obtained with the pE1A and S4A antigens, which also generate antibodies that do not cross-react with GnRH-II.
In this study we show that the antibody response after immunization can be directed by appropriatly engineered antigens in order to prevent the occurrence of possible harmful side effects caused by cross-reaction of GnRH-I directed antibodies with other GnRH isoforms.

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Chapter 6

Performance of male pigs immunized against GnRH is related to the time of onset of biological response

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ABSTRACT

In this study, the performance of male pigs immunized against GnRH was determined in relation to the onset of their biological response to the immunization. Pigs were immunized at 9 and 17 weeks of age and were housed in a pen together with both a surgically castrated and an intact boar littermate. Feed intake was restricted to 2.8 to 3.2 times maintenance requirement for energy. Animals were weighed weekly and slaughtered at 108 kg bodyweight (BW). Depending on the time of onset of the response after immunization in terms of biological effects, immunized pigs were retrospectively grouped into two categories. One category consisted of the immunized pigs, which had undetectable or low levels of LH and testosterone at the time of booster immunization, 'early' responding immunocastrates (E-IM, n = 8), while the 'late' responding immunocastrates (L-IM, n = 7) had substantial LH and testosterone levels at that time. This dichotomy of the response to immunization was also reflected in testis weight, with 17 g and 40 g for E-IM and L-IM pigs, respectively. At slaughter, testis size and testis weight were reduced ($P < 0.001$) in the immunocastrated pigs as compared to the intact boars. Androstenone concentrations in backfat of all immunocastrated pigs were undetectable. Growth performance, i.e. average daily gain (ADG, g gain/day) and feed efficiency (FE, g gain/kg feed), was better in boars and L-IM pigs than in surgical castrates and E-IM pigs ($P < 0.05$). ADG and FE did not differ between E-IM pigs and the surgical castrates, but intact boars performed better than L-IM ($P < 0.02$). There were no significant differences in carcass quality (backfat thickness and meat percentage) between boars and surgical castrates at slaughter. However, for both characteristics L-IM pigs and intact boars performed better ($P < 0.03$) than E-IM pigs. Thus, growth performance in L-IM pigs is better than in either E-IM pigs or surgical castrates.

1. INTRODUCTION

Surgical castration of male piglets is common practice, in order to prevent the occurrence of boar taint in pork. Surgical castration, however, has disadvantages. It is a painful and animal unfriendly practice (McGlone and Hellman, 1988), and results in doubling of the incidence of chronic inflammations at slaughter as compared to boars (De Kruijf and Welling, 1988). Surgical castration also affects growth performance (Walstra and Vermeer, 1993; Stamer et al., 1993), and increases the excretion of nitrogen and phosphate in the manure (Vermeer et al., 1992).

Immunocastration, i.e. active immunization against GnRH, may offer an alternative for surgical castration (Meloen et al., 1994; Oonk et al., 1998; Hennessy et al., 1997). In boars that are immunized against GnRH, the concentrations of androstenone and skatole (both contributing to boar taint, Matthews et al., 2000) are at the level of surgical castrates, resulting in taint-free pork (Hennessy et al., 1997).

Immunocastration preferably is performed in the finisher phase, in order to utilize maximally the boar-type growth during the period before the onset of immunocastration (Bonneau et al., 1994). However, immunocastration shortly before slaughter does not allow discrimination between immunocastrated boars and intact boars by simply estimating the testis size. In our study, therefore, we applied immunization at 9 and 17 weeks of age, which has shown to meet the criteria of visible reduced testis size (Oonk et al., 1995). In previous experiments we observed a considerable variation between animals in their response to the immunizations. We hypothesized that a more boar-like performance can be expected in GnRH-immunized boars with a 'late' response, as determined by substantial LH and testosterone levels still being present at the time of second immunization, than in GnRH-immunized boars with an 'early' response or in surgical castrates.

2. MATERIALS AND METHODS

2.1 Animals

The experimental protocol describing the management, surgical procedures and animal care, was reviewed and approved by the Dutch Committee on Animal Care and Ethics. Forty-eight piglets from sixteen litters of Dutch commercial crossbred pigs (Dutch Landrace x Finnish Landrace) x (Large White) were selected in the first week after birth. From each litter three male piglets were chosen, one of which was surgically castrated between 8 and 18 days after birth, whereas one served as an intact control and one was immunized at 9 and 17 weeks of age. The three piglets of a litter were randomly assigned to the treatment groups.

Two days before the start of the experiment, the three littermates were allocated to the same pen, with 3 pigs per pen. The pens consisted of two sections: one section for individual

feeding and a group-section with a half-slatted floor for lying and dunging, in which section also additional water was supplied. The animals were fed individually two times a day. Feed intake was restricted to 2.8 times ME_m (equals 418 kJ ME/$W^{0.75}$) at the start of the experiment and was gradually increased to 3.2 times ME_m. From the start of the experiment at 23 kg BW until the pigs weighed 45 kg, a starter diet was fed, and from 45 kg BW to slaughter a grower diet was supplied. The feed was offered together with water at a ratio of 1: 2.5 in the trough, while the pigs had free access to water in the group-section. The diets were composed of feedstuffs commonly used in The Netherlands (mainly barley, soybean meal and tapioca). They were formulated to contain 110% of the current Dutch recommendations of amino acids for slaughter pigs in order to allow the boars to fully utilize their growth potential (CVB, 1997). During manufacturing of the diets, triplicate samples were taken and analyzed according to the methods described by AOAC (1984).

2.2 Experimental design and treatments

One intact male piglet out of each triplet was immunized at 9 weeks of age (d 0) and at 17 weeks of age (8 weeks post vaccination, 8 wk) by i.m. injection at the left and right side of the neck region, respectively. The vaccine consisted of 1 mg synthetic GnRH-tandem peptide in 2 mL Freund's complete adjuvant emulsion (Oonk et al., 1998). For the second immunization Freund's incomplete adjuvant was used. The remaining intact male piglet of each triplet was not injected.

The immunized pigs were retrospectively divided into two categories, based on their LH and testosterone levels at the time of booster immunization (i.e. at 8 weeks after the first immunization). Immunized pigs were defined as 'early' responders (E-IM) when both LH and testosterone concentrations at 8 wk were low. Testosterone concentrations should be similar to those of surgical castrates (< 0.40 pmol/mL) and LH should be less than 0.80 ng/mL. Immunized pigs that had testosterone concentrations at 8 wk exceeding the level of surgical castrates were defined as 'late' responders (L-IM). LH levels of the L-IM pigs were variable and ranged between 0.63 and 1.95 ng/ml. The E-IM and the L-IM group consisted of 8 and 7 pigs, respectively. One immunized pig and its intact and surgically castrated male littermates were excluded from the analysis of the results of this experiment, because the immunized pig did not respond to the immunizations. In contrast to the other immunized pigs, this pig exhibited substantial serum LH and testosterone levels throughout the experiment and the weight of its testes was similar to that found in intact boars.

2.3 Measurements

Animals were weighed weekly in order to determine the amount of feed that should be supplied. Blood samples were taken at d 0, 8 wk, 12 wk and one day before slaughter. Blood samples were taken via puncture of the vena jugularis and kept overnight at 4°C. The

next day, serum was obtained by centrifugation (2,000 g, 15 min.). Serum samples were stored at –20°C until assayed. Testis size was determined by measuring testis length using a vernier calipers at each day of blood sampling; mean values of the two testes were recorded. At 90 kg BW and at one day before slaughter ultrasonic backfat measurements were performed. Backfat thickness was measured at 4 positions on the left side and at 4 positions on the right side of the median. Measurements were performed according to the method described by Walstra (1987). Pigs were slaughtered one week after they weighed at least 102 kg. Feed was withheld the morning before slaughter. Animals were slaughtered according to the Dutch regulations for a commercial slaughterhouse. Testes were removed and testis weight was recorded and averaged for each animal. At slaughter, warm carcass weight was determined. Meat percentage was measured by the Hennessy Grading Probe (HGP) (Walstra, 1987). This method is routinely used in the Dutch slaughterhouses to classify pigs. Backfat samples were taken from the shoulder region of each carcass and stored at –20°C until analysis.

2.4 Hormone analyses

GnRH antibody titers were determined by studying the binding of serial dilutions of pig sera to [^{125}I]GnRH as described by Meloen et al. (1994). Titers were expressed as percentage binding of [^{125}I]GnRH at a given serum dilution. LH concentrations were determined with a RIA as described by Van de Wiel et al. (1984). Porcine LH for use both as a reference preparation and for iodination, was purchased from UCB, Brussels (code H028/H pLH). Inter-assay variation was 9.1%, intra-assay variation was 10.6% and the detection limit was 0.14 ng/ml. Serum testosterone levels were determined using a Coat-a-Count kit (DPC laboratories, Los Angeles, CA) with a detection limit of 0.14 pmol/mL. The coefficients of variation for intra- and inter-assay variability were 4 to 6% and 7 to 11% respectively. The amount of androstenone in backfat was determined using an ELISA (R-Biopharm, Darmstad, Germany as distributed by Ridascreen, Almere, The Netherlands). The detection limit of this ELISA was 0.1 µg/g and intra- and inter-assay coefficients of variation were 0.4 to 4.5 % and 2.0 to 3.9 % respectively.

2.5 Statistical analyses

The experiment was performed in two replications, with 8 and 7 litters per replication, and there were two strata of variation, namely litters within replications and pigs within litters. Growth performance, carcass traits, testis weight, LH and testosterone data were analysed by Restricted Maximum Likelihood methods, according to the following statistical model:
$Y = \mu + rep + litter + group + error$,

where μ = overall mean, rep = replication, litter = random effect of litter within replications, group = effect of treatment with a subdivision of immunocastrates into 'early' and 'late' responders (boar, 'early' responder, 'late' responder, surgical castrate), error = random error contribution with mean 0 and variance σ^2.
Since we were particularly interested in antibody titers, LH levels, testosterone levels and testis size at two specific timepoints after immunization, namely at booster immunization and at slaughter, no special methods were applied with respect to repeated measurements. In those cases where an overall Wald's test yielded a significant difference between groups, homogenous subsets of groups are formed by means of least significant differences and indicated in the usual way in the tables concerned. Testis weight, LH and testosterone levels were log-transformed to reduce heterogeneity of variance. For antibody titers, only two groups were involved in the statistic analysis, so that a Student's t-test could be applied. All significance levels were set at 5%. The REML-analyses were performed with Genstat (Genstat 5 Committee, 1993, release 3, Reference manual. Clarendon Press, Oxford, UK) and the non-parametric analyses with StatXact (StatXact 4 for Windows, 1998, Cytel Software Corporation, Cambridge, USA).

3. RESULTS

3.1 Immunological and endocrine responses

Eight weeks after the first immunization, significant antibody titers, ranging from 12 to 45 % binding of [^{125}I]-GnRH in 1/2000 serum dilution, were present in 14 out of 15 immunocastrated pigs. At slaughter, high antibody titers were present in all immunocastrated pigs. There was no significant difference in antibody titers among E-IM and L-IM pigs, although antibody titers were nummerically higher for E-IM pigs at both timepoints (Table 1).
At 8 wk, LH concentrations in the immunocastrated pigs were lower ($P < 0.001$) than in both the intact boars and surgically castrates (Table 1), whereas LH levels in the L-IM pigs were higher ($P < 0.001$) than in E-IM pigs. Testosterone levels at 8 wk were lower ($P < 0.05$) in both L-IM and E-IM pigs than in the intact boars, but were substantially higher ($P < 0.001$) in L-IM (Table 1) than in E-IM pigs. There was no difference in testosterone levels at 8 wk between E-IM and surgical castrates. At slaughter, LH and testosterone levels were lower in both E-IM and L-IM pigs ($P < 0.05$) than in intact boars. LH and testosterone levels at slaughter of the non-responding immunized boar were 0.94 ng/mL and 6.74 pmol/mL respectively, which is in the range of intact boar values. The difference in biological response, i.e. testosterone and LH at 8 weeks, did not depend on the BW of the immunized pigs at time of first immunization. BW was 23.1 kg and 23.0 kg for L-IM and E-IM pigs, respectively.

Table 1. GnRH antibody titers, LH and testosterone concentrations of boars (n=15), immunocastrates (n=15) and surgical castrates (n=15). Immunocastrates were allocated to two groups based on the LH and testosterone levels at time of booster immunization (8 wk). Immunocastrates with serum testosterone concentrations on the level of surgical castrates (< 0.40 pmol/mL) and LH concentrations at the level of immunocastrated boars (< 0.80 ng/mL) were allocated to the 'early' group (n=8), while the remaining immunocastrates, which all exhibited testosterone concentrations exceeding the level of surgical castrates and variable LH concentrations were allocated to the 'late' group (n=7)[a].

Item	Boars	'Late' Immunocastrates	'Early' Immunocastrates	Surgical Castrates
GnRH antibody titers [b]				
- 8 wk [c]	ND	19.7 ± 5.9	29.4 ± 2.5	ND
- At slaughter	ND	40.7 ± 5.1	46.5 ± 1.9	ND
LH, ng/mL				
- 8 wk [c]	1.63^{f} ± 0.15	1.11^{e} ± 0.17	0.53^{d} ± 0.05	2.85^{g} ± 0.18
- At slaughter	1.12^{e} ± 0.11	0.66^{d} ± 0.04	0.58^{d} ± 0.01	2.10^{f} ± 0.21
Testosterone, pmol/mL				
- 8 wk [c]	11.4^{f} ± 1.51	5.47^{e} ± 1.04	0.25^{d} ± 0.03	0.21^{d} ± 0.01
- At slaughter	8.22^{f} ± 1.46	0.41^{e} ± 0.10	0.22^{de} ± 0.01	0.22^{d} ± 0.01

[a] Values are reported as means ± SEM
[b] Percentage binding of [^{125}I]GnRH by a 1:2000 serum dilution.
[c] 8 wk = time of booster immunization, 8 weeks after the first vaccination; ND = not detectable
[d, e, f,g] Means within a row without a common superscript letter differ ($P < 0.05$)

3.2 Testis function

The differences in LH and testosterone levels between E-IM and L-IM pigs at the time of booster immunization corresponded with the differences in testes size at that time. Testes length was smaller ($P < 0.001$) in E-IM pigs than in L-IM pigs (4.1 and 5.7 cm, respectively), whereas similar testis length was found in L-IM pigs and intact boars (Figure 1). At slaughter, testes of both E-IM and L-IM pigs were smaller ($P < 0.001$) than testes of the intact boars, while testes were smaller ($P < 0.001$) in E-IM than L-IM pigs. This corresponded with a lower ($P < 0.001$) testis weight for the E-IM pigs than for L-IM pigs (17 g and 41 g, respectively). Testis weight of the intact boars was 163 g. When analysed for all immunocastrated pigs, the correlation between testosterone concentrations at 8 weeks and the size of the testis ($r = 0.85$; $P < 0.01$) was much higher than the correlation between GnRH antibody titer and testis size ($r = -0.42$; $P < 0.05$).

3.3 Androstenone

Androstenone concentrations in backfat of all immunocastrated and surgically castrated pigs were below the detection limit of the assay (<0.1 µg androstenone/g backfat). In the intact boars, androstenone levels in backfat ranged from undetectable to 1.25 µg/g and mean value was 0.48 µg/g. Androstenone concentration in the backfat of the non-responding immunized pig was 0.19 µg/g.

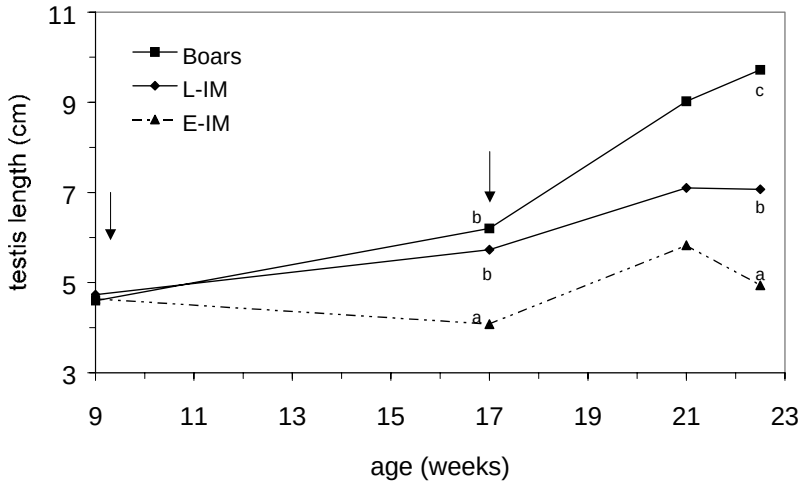

Figure 1. Time course of changes in average testis size of boars (n=15) and immunocastrates (n=15). Immunocastrates were allocated to two groups based on LH and testosterone levels in the serum at time of booster immunization (8 wk). Immunocastrates with serum testosterone concentrations on the level of surgical castrates (< 0.40 pmol/mL) and LH concentrations at the level of immunocastrated boars (< 0.80 ng/mL) were defined as 'early' responding immunocastrates (E-IM, n=8), and the remaining immunocastrates, which all exhibited testosterone concentrations exceeding the level of surgical castrates and variable LH levels, were defined as 'late' responding immunocastrates (L-IM, n=7). Arrows indicate times of immunization.
[a,b,c] Means with different superscript measured at the same timepoint differ ($P < 0.001$).

3.4 Growth performance

ADG for the period between the first (d 0) and the second immunization, 8 weeks later, was not influenced by treatment ($P = 0.17$), although a difference between boars and E-IM was observed. For the second period (8 wk to slaughter) and the whole experimental period (d 0 to slaughter) differences among treatments were more pronounced (Table 2). Boars grew

faster than immunocastrates (E-IM and L-IM) or surgical castrates ($P < 0.05$) between 8 wk and slaughter. For the whole experimental period ADG of the L-IM pigs was less than that of the intact boars ($P = 0.02$) and higher ($P < 0.05$) than ADG of the E-IM pigs or surgical castrates.
Boars had a higher FE ($P < 0.01$) than surgical castrates between d 0 and 8 wk, while immunocastrates were intermediate (Table 2). After the second immunization, FE of both, E-IM and L-IM pigs, was similar to the surgical castrates and lower than FE of the boars ($P < 0.01$). Overall, FE was higher in L-IM pigs than in surgical castrates or E-IM pigs ($P < 0.05$), but lower than in boars (P = 0.001)

Table 2. Growth performance of boars, immunocastrates and surgical castrates. Immunocastrates were allocated to two groups based on the LH and testosterone levels at time of booster immunization (8 wk)[a].

Item	Boars	'Late' Immuno-castrates	'Early' Immuno-Castrates	Surgical Castrates	SEM
ADG (g/d)					
Day 0 – 8 wk[b]	766[d]	763[cd]	744[c]	752[cd]	9
8 wk – slaughter	1206[d]	1135[c]	1086[c]	1103[c]	19
Day 0 – slaughter	941[e]	913[d]	885[c]	890[c]	8
Feed Efficiency, gain (g)/feed (kg)					
Day 0 – 8 wk	522[d]	516[cd]	507[cd]	504[c]	5
8wk- slaughter	434[d]	407[c]	392[c]	397[c]	6
Day 0 – slaughter	473[e]	455[d]	441[c]	444[c]	4

[a]Values are reported as means. For further explanation, see Table 1.
[b] wk = weeks post first vaccination
[c, d, e] Means within a row without a common superscript letter differ ($P < 0.05$)

3.5 Carcass characteristics

Backfat thickness at 90 kg BW, was smaller for L-IM pigs than for E-IM pigs or surgical castrates ($P < 0.01$) (Table 3) and did not differ from intact boars. At slaughter, backfat thickness of the boars and L-IM pigs was smaller than for the E-IM pigs ($P < 0.03$), but was not different from the surgical castrates anymore. Meat percentage was inversely proportional to backfat thickness at slaughter, which was also reflected in a higher meat percentage of the boars and L-IM pigs ($P < 0.01$) as compared to E-IM pigs.

4. DISCUSSION

In the present study, active immunization against GnRH reduced serum LH and testosterone to undetectable or low levels in 50 % of the pigs (E-IM) at the time of booster immunization. Due to the rapid immunocastration effect in these pigs, testicular function was affected at that time, as was evident from smaller testes as compared to the L-IM pigs and the intact boars. Biological responses after immunization against GnRH are known to depend on the ability of the elicited antibodies to neutralize native GnRH. However, in this study GnRH antibody titers at the time of booster immunization were not different between the E-IM and the L-IM pigs. This results in a low correlation between GnRH antibody response and testis size at that time (r = -0.42). As suggested by Ghosh and Jackson (1999), the observed biological effect may correlate better with the length of the time period during which the antibodies are present than with the GnRH antibody response at a certain timepoint. In addition, the biological effects of antibody titers also depend on the affinity of the antibodies. As a result of differences in affinity, biological effects among animals may differ even when antibody titers are similar (Quesnell et al., 2000).

Table 3. Carcass characteristics of boars, immunocastrates and surgical castrates. Immunocastrates were allocated to two groups, based on the LH and testosterone levels at time of booster immunization (8 wk)[a].

Item	Boars	'Late' immuno-castrates	'Early' immuno-Castrates	Surgical castrates	SEM
Backfat (mm)					
- At 90 kg BW	11.2^{bc}	10.6^{b}	12.0^{c}	11.7^{c}	0.3
- At slaughter	13.0^{b}	12.6^{b}	14.3^{c}	13.5^{bc}	0.4
BW at slaughter (kg)	110.3^{d}	109.3^{cd}	107.5^{bc}	106.7^{b}	0.9
Carcass wt (kg)	83.5^{c}	82.2^{bc}	81.9^{bc}	81.2^{b}	0.8
Dressing %	75.5^{bc}	75.2^{b}	76.2^{c}	76.1^{c}	0.3
Meat %	55.5^{c}	56.2^{c}	53.6^{b}	54.9^{bc}	0.5

[a] Values are reported as means. For further explanation, see Table 1.
[b,c,d] Means within a row without a common superscript letter differ ($P < 0.05$)

Androstenone levels were undetectable in the backfat of both 'early' and 'late' immunocastrated pigs, demonstrating that a complete clearance of androstenone from the fat tissue was achieved, a process which is known to require a period of at least 3 weeks (Claus et al., 1994). In the present study, androstenone levels exceeded the threshold value of 0.5 µg androstenone/g backfat in 50% of the intact boars, whereas the average androstenone value was 0.48 µg/g. In other studies, similar figures were found (Bonneau et al., 1994; Hennessy et al., 1997; Walstra et al., 1999).

Mean testis weight of the intact boars was relatively low (163 g) compared to results of other studies (Falvo et al., 1986; Meloen et al., 1994). This may be due to the relatively early age of the pigs at slaughter, which was 158 days. At this age, testis weights are rapidly increasing (FlorCruz and Lapwood, 1978; Van Straten and Wensing, 1978; Lunstra et al., 1986). Most likely, the majority of the pigs in our study did not reach this stage. Despite the young age of the pigs, a distinct difference in testis size at slaughter could be observed between immunocastrated pigs and boars (Figure 1). Testes of the E-IM and L-IM pigs were 50% and 25% smaller, respectively, than testes of the boars. The difference in testis size between boars and immunocastrated pigs became also visible by the outside appearance of the scrotum. Immunocastrates exhibited a flat scrotal sac, while the scrotum of the boars had a more bulbous appearance. This enables us to distinguish immunocastrated pigs from intact boars by the size of the testis and the appearance of the scrotum.

In the present study, the results for ADG under restricted feeding conditions corroborate with literature values (Walstra and Vermeer, 1993). However, differences in FE between intact boars and surgical castrates were markedly smaller than expected. Nevertheless, FE in the L-IM pigs was higher than in the E-IM pigs and the surgical castrates ($P < 0.05$). An improved, more boar-like FE of immunocastrated pigs vs surgical castrates has also been reported by Bonneau et al. (1994) and Zeng et al. (2002). In both studies, however, pigs were given ad libitum access to feed. It is well known that under ad libitum feeding conditions surgical castrates eat more than boars, subsequently resulting in a less efficient FE of the surgical castrates as compared to boars. In this way the difference in FE between intact boars and surgical castrates is enlarged, which makes it more likely for FE of the immunocastrates to be in between FE of boars and surgical castrates. In our previous study (Zeng et al., 2002), this indeed was observed, despite the fact that most of the immunocastrated pigs exhibit hormone profiles similar to the E-IM pigs in the present study. In the study of Bonneau et al. (1994) the high FE of the immunocastrates, may not only be due to the ad libitum feeding regimen, but also to the fact that the second immunization was applied only two weeks before slaughter and that the immune response of some immunized pigs was rather low. The higher FE in the L-IM pigs than in the E-IM and the surgical castrates, was reflected also in a higher ADG for the L-IM pigs ($P < 0.05$). A comparable effect was observed under ad libitum feeding conditions (Zeng et al., 2002), where both ADG and FE tended to be higher in immunocastrates than in surgical castrates. There were no significant differences between intact boars and surgical castrates in meat percentage and backfat thickness at slaughter. This can most likely be explained by the fact that feed intake of the pigs was restricted, and therefore, the surgical castrates deposited less fat, resulting in smaller differences for meat percentage and backfat thickness than expected under ad libitum feeding conditions (Walstra et al., 1977; Zeng et al., 2002). This could also

be the reason that the L-IM pigs did not differ from the surgical castrates at slaughter. However, backfat thickness at 90 kg BW was less in the L-IM pigs than in surgical castrates, and both meat percentage and backfat thickness at slaughter were better in the L-IM pigs than in the E-IM pigs. In conclusion, growth performance is better in the L-IM pigs than in the E-IM pigs and the surgical castrates, whereas carcass quality was better in L-IM pigs than in E-IM pigs. Thus, 'late' immunocastration could be an alternative for surgical castration, which not only prevents the occurrence of boar taint, but also has the advantage of improved growth performance.
A fully effective ‘late’immunocastration, which allows discrimination between immunocastrates and boars at slaughter, can be achieved by application of a vaccine that comprises an immunogenic GnRH antigen in combination with an adjuvant that generates a rapid and high secondary immune response. In this case the first immunization, which is intended to prime the immune system, preferably elicites a low or moderate antibody response, without complete GnRH neutralizing activity. The vaccine formulation used in our previous studies (Oonk et al., 1998; Turkstra et al., 2002; Zeng et al., 2002), i.e. D-Lys^6-GnRH tandem dimer ovalbumin conjugate in Specol adjuvant, does not meet these criteria. It induces a biological effect in a majority of the treated boars already after a single immunization. Nevertheless, even with mineral oil adjuvants, a second immunization is needed to elicite a GnRH neutralizing antibody response in all treated animals. In practice, ‘late‘ immunocastration requires an effective second immunization, administered at least 5 weeks before slaughter in order to obtain a distinct difference in testis size necessary to distinguish immunocastrated boars from untreated boars. The use of vaccines that are able to establish ‘late’ immunocastration, due to a strong secondary response, most likely will result in improved performance of the immunized pigs.

5. IMPLICATIONS

In this study, it was demonstrated that performance of boars which were immunocastrated by active immunizion against GnRH, depends on the time of onset of the biological response. Performance of immunized boars that were already immunocastrated before the booster immunization ('early'), was less than performance of immunized boars which were effectively immunocastrated after the booster immunization ('late') . Growth performance was also more boar-like in 'late' immunocastrated than in surgically castrated pigs. Moreover, both 'early' as well as 'late' responding immunocastrates could easily be distinguished from untreated boars by the size of the testis and the outward appearance of the scrotum. Our results indicate that vaccination against GnRH, especially with vaccines that are able to establish ‘late’ immunocastration, could be a practical and profitable alternative to surgical castration in pigs.

6. ACKNOWLEDGEMENTS

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Chapter 7

Effects of GnRH immunization in sexually mature pony stallions

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ABSTRACT
Immunization against gonadotrophin releasing hormone (GnRH) was studied as an alternative for the commonly used surgical castration in stallions. Two GnRH vaccines comprising non-mineral oil adjuvants were evaluated for their potential to induce high antibody titers directed against GnRH and subsequent effects on reproductive characteristics. Twelve sexually mature male hemicastrated Shetland ponies were assigned to 3 groups. Group 1 and 2 were injected with 1 mg peptide equivalent of G6k-GnRH-tandem-dimer conjugated to ovalbumin (OVA) in CoVaccine^{TM} HT adjuvant (GnRH/CoVaccine) and in Carbopol (GnRH/Carbopol) respectively, and group 3 was injected with CoVaccine^{TM} HT adjuvant without antigen (controls).
After immunization no adverse effects were observed with respect to the injections sites or general health. Two weeks after the second vaccination antibody titers against GnRH increased rapidly in all animals of the GnRH/CoVaccine group, at the same time reducing serum testosterone levels maximally for the further duration of the experiment. In the GnRH/Carbopol group antibody responses and effects on testosterone levels were intermediate in two stallions and not apparent in the remaining stallions of this group. Semen evaluation showed that from two weeks after the second immunization onwards, sperm motility was affected in all stallions treated with GnRH/CoVaccine and one stallion treated with GnRH/Carbopol. Seven weeks after the second immunization, no semen could be collected from two stallions, one of each group, due to suppressed libido. Histological examination of the testes, 15 weeks after the initial immunization, demonstrated reduction in seminiferous tubuli diameters in all stallions of the GnRH/CoVaccine group and one stallion of the GnRH/Carbopol group. Furthermore, spermatogenesis was extremely disorganized in these stallions, as indicated by absence of the lumen in the seminiferous tubules, the absence of spermatozoa and spermatids in the tubular cross-sections and the impossibility to determine the stage of the tubular cross sections. Testis size was also substantially reduced in 3 out of 4 stallions treated with GnRH/CoVaccine.
The results demonstrate that two immunizations with G6k-GnRH-tandem-dimer OVA conjugate in a suitable adjuvant such as CoVaccine^{TM} HT caused a rapid and complete reduction of serum testosterone levels in sexually mature stallions, subsequently leading to reduced sperm motility and affected testis function, while no adverse reactions were observed after immunizations.

1. INTRODUCTION

Worldwide, every year a large number of stallions are surgically castrated, mainly to prevent aggressive and unwanted sexual behaviour (Moll et al., 1995). This commonly used castration method causes complications in more than 5% of the treated stallions (Keller and Hartmann, 1996). Castration of cryptorchid stallions has an even higher risk for complications (Searle et al., 1999). Therefore a non-surgical castration method without the risk of side effects would be desirable.

Several hormone treatments have been proposed in order to reduce libido and aggresive behaviour, but efficacy appeared to be unsatisfactory and frequent administration was required (Roberts and Beaver, 1987, Hinojosa et al., 2001). A practical alternative may be active immunization against GnRH. This approach has been evaluated in many species in an experimental set-up and in most cases it has proven to be an useful alternative for the current surgical castration methods (Thompson, 2000). For various reasons, however, only a few vaccine formulations used in these studies are considered to be suitable for use in practice.

Although in male horses several studies have described the effects of GnRH vaccination, a suitable vaccine for practical use has not been described sofar. Schanbacher and Pratt (1985) were the first to report the succesful vaccination of a cryptorchid stallion against GnRH: blood testosterone levels were reduced and the stallion behaved docile. In that study, however, Freunds adjuvant was used as an adjuvant. Local reactions at the site of injection were observed in colts after immunization with a mineral oil based GnRH-vaccine (Dowsett et al., 1991). All treated stallions showed depressed testosterone levels and an affected testis function. As the occurrence of adverse local reactions after vaccination will not be accepted by the horse owners, the vaccine preferably should contain an adjuvant which does not induce injection site reactions. A GnRH vaccine containing a water-soluble adjuvant was tested in young stallions (Dowsett et al. 1996). After two to three immunizations a 'castration' effect was observed lasting for six months after the first vaccination, as determined by a decrease in testosterone concentration. Treated stallions exhibited no libido in the presence of a mare in oestrus, semen could not be collected and no sexual agressive behaviour was observed. This study also demonstrated that GnRH immunization is reversible. When immunizations were not repeated, a recovery of the testis function was observed and libido returned to normal again. Such a reversible 'castration' method can save the reproductive capacity of stallions for later when offspring of these males is desired. Unfortunately, this vaccine has not been applied in sexually mature stallions, which most likely will be the most important treatment group. In a recent study, three adult stallions were treated with a GnRH vaccine with a commercially available 'horse adjuvant' Equimune (Malmgren et al., 2001). However, this vaccine failed to induce high

antibody titers in one of the three stallion, despite the fact that four booster injections were given. Moreover, local tissue reactions were observed in all treated stallions.
The aim of the present study was to investigate the efficacy and safety of two new immunization protocols employing the highly effective G6k-GnRH-tandem- dimer-OVA conjugate (Oonk et al., 1998) in non-mineral oil adjuvants in sexually mature pony stallions.

2. MATERIALS AND METHODS

2.1 Vaccine formulations

The GnRH-tandem peptide with amino acid Glycine on position 6 and 16 substituted by a D-Lysine, and a C-terminal Cysteine added, was synthesized as reported previously (Oonk et al., 1998). After purification, the peptide was dimerized via its C-terminal Cysteine purified again and conjugated to OVA, as described by Oonk et al. (1998). The GnRH conjugate (G6k-GnRH-tandem-dimer-OVA conjugate) contained 0.5 mg G6k-GnRH-tandem-dimer peptide per mg OVA, as determined by comparative amino acid analysis. Two vaccins on the basis of this GnRH conjugate were formulated. The first vaccine (GnRH/CoVaccine), was prepared by mixing the adjuvant, CoVaccine^{TM} HT kindly provided by CoVaccine BV (Lelystad, The Netherlands) with an equal volume of phosphate buffered saline (PBS), containing the antigen. For the second vaccine (GnRH/Carbopol), 8 mg Carbopol 934 PH (BF Goodrich, Cleveland, Ohio, USA) per ml water, was mixed with an equal volume of PBS containing the antigen. CoVaccine^{TM} HT diluted with PBS without antigen was used as a control.

2.2 Animals

Twelve adult male 4 year old Shetland ponies, which were hemicastrated three weeks before the start of the experiment by removing the left testis, were assigned to three groups (GnRH/CoVaccine, GnRH/Carbopol and controls) of 4 animals each. The stallions had free access to water, grass and straw during the period from June to September and from September untill the end of the experiment (October 15) grass was replaced by hay and silage. They were group housed and kept indoors under natural photoperiod during the entire experimental period.

2.3 Immunization

The stallions were immunized during the reproductive season on June 30 and received a booster immunization 6 weeks later. Two ml vaccine, containing 1 mg G6k-GnRH-tandem-dimer peptide equivalent of the conjugate, or the control vaccine were given intramuscularly at two sites in the pectoral muscle. During three days after immunization,

the injection sites were examined for adverse reactions and the general condition of the ponies was evaluated.

2.4 Blood samples, assays and measurements

Animals were bled every two weeks by puncturing of the jugular vein for determination of GnRH antibody titers and testosterone concentrations. Serum was prepared by centrifugation at 1400g for 15 minutes. Samples were stored at -20°C until assayed. Antibody titers against GnRH were determined with a radio-immuno-assay (RIA) as described by Meloen et al. (1994). An amount of 50 µl antiserum diluted 1:1000 in PBS with 0.4 % bovine serum albumine (BSA) was allowed to bind with iodinated GnRH (Amersham Pharmacia Biotech, Buckinghamshire, England) in 50 µl PBS with 0.4% BSA. After incubation for two days at 4°C, the iodinated GnRH bound to the antibodies was separated from the unbound using dextran-coated charcoal. The supernatant was counted and the percentage of iodinated GnRH bound by the antibodies in the 1:2000 diluted serum was calculated.

Testosterone levels in serum were measured with the a Coat-a-Count kit from DPC Laboratories (Los Angeles, CA, USA).

The size of the testis was determined with vernier calipers at time of first and second immunization and 14 weeks after the initial immunization.

Body weight of the stallions was measured at the start and the end of the experiment.

2.5 Semen collection and evaluation

The fertility status of the colts during the experiment was determined by evaluation of the semen at first immunization and 2, 4, 6, 8, 10 and 13 weeks thereafter. A mare in estrus was used to initiate sexual behaviour. Semen collection and evaluation were performed as described by Van der Meer et al. (1999). The volume of the ejaculate was recorded, and sperm motility and concentration were estimated.

2.6 Histological evaluation

The remaining testis of the hemicastrated stallions was surgically removed at 15 weeks after the initial vaccination and used for histological examination. Two to three cross sectional slices of each testis were fixed in 4% buffered formalin for 8 h at room temperature. This was followed by a post-fixation in Bouin's solution (85 ml 0.9% picric acid, 15 ml 40% formalin and 5 ml acetic acid) for 18 h at room temperature. The tissues were embedded in paraffin, and 5 µm sections were cut and stained with Mayer's heamatoxylin and eosin.

The average diameter of the seminiferous tubuli was determined for each stallion by measuring 35 to 70 tubuli per testis, using a Nikon Optiphot 2 microscope equiped with a

Leica image analysis program. Changes in tubuli diameter were determined by comparing the average tubuli diameter of the 'pre-immunization' testis with the tubuli diameter of the testis obtained after castration at 15 weeks after the initial immunization.

Table 1. The criteria for the scores from 1 to 10 according to Johnson (1970), modified for the use in the horse.

Johnson score	Criteria
10	Complete spermatogenesis with many spermatozoa. Germinal epithelium organised in regular thickness leaving an open lumen, or stage I of the seminiferous cycle, with sufficient round spermatids.
9	Many spermatozoa present but germinal epithelium disorganized, with marked sloughter or obliteration of the lumen.
8	Only few spermatozoa (<5-10) present in a tubular cross-section.
7	No spermatozoa but many spermatids present.
6	No spermatozoa and only a few spermatids (<5-10) present.
5	No spermatozoa, no spermatids but several or many spermatocytes present.
4	Only few spermatocytes (<5) and no spermatids or spermatozoa present.
3	Spermatogonia are the only germ cell type present.
2	No germ cells but only Sertoli cells present.
1	No cells in tubular cross-section visable.

In at least two different tissue blocks per testis two to three sections were traversed randomly and 100 round tubular cross-sections were studied using the Johnson score method (Johnson, 1970). The Johnson score gives a score of 1-10 according to the presence or absence of germ cell types (see Table 1). Since the Johnson score was developed to quantify human spermatogenesis, a minor modification was introduced to account for the difference in the seminiferous tubule architecture between humans and horses. In man several stages of the spermatogenic cycle can be observed in one tubular cross-section, and thus when spermatogenesis proceeds normally, elongated spermatids should be present in each cross-section. In the horse, as in other mammals, each tubular cross-section normally contains only one stage of the spermatogenic cycle. Consequently in the stage where sperm is released from the epithelium (stage I in the horse), no elongated spermatids can be detected (Johnson et al., 1990). In this case the Johnson score would be 7 for a normal tubule. To overcome this problem, a cross-section in stage I was given a score of 10 when enough normal round spermatids, characteristic of stage I, were present. All other stages

were classified according to the regular Johnson score. Subsequently, the average mean Johnson score per horse was calculated.

2.7 Statistical Analysis

Mean values for the tubular diameters and Johnson scores were calculated and expressed ± SD. Semen characteristics, i.e. sperm motility, semen volume, sperm concentration and total sperm counts, were averaged for 8, 10 and 13 weeks after the first immunization, representing the 'post booster' values. Changes throughout the experiment were expressed as the slope, determined by regression analysis, of the values at 0, 2, 4, 6, 8, 10 and 13 weeks after the first vaccination.

Means were considered to be statistically significant when $P < 0.05$ using the Mann Whitney U test.

3. RESULTS

The treatments had no effects on body weight. Weight gain of the stallions during the experiment ranged between 2 and 25 kg. The vaccines were well tolerated. After immunization only a transient rise of body temperature (>38°C) could be detected in some of the stallions of all three groups. No adverse reactions at the site of injection were noticed and no behavioural abnormalities were observed after immunization.

Antibody responses after the first vaccination, were low in the stallions of both GnRH treated groups (GnRH/CoVaccine and GnRH/Carbopol). Two weeks after the booster vaccination, antibody titers became high, ranging from 56 - 76 % binding of iodinated GnRH in a RIA, in the stallions treated with GnRH/CoVaccine (Figure 1). Antibody titers remained at a high level in this group, resulting in undetectable testosterone levels two weeks after the second vaccination. Testosterone levels remained undetectable until the end of the experiment (Figure 2). In the GnRH/Carbopol group, only two stallions (794 and 795) responded to the second vaccination with significant antibody titers (24 - 27 % binding of iodinated GnRH), although these antibody titers were lower than those of the GnRH/CoVaccine treated stallions. Maximum binding of iodinated GnRH in the remaining two stallions of GnRH/Carbopol group were less than 2% at the tested serum dilution (1:2000). One of the responding stallions of the GnRH/Carbopol group, stallion 794, showed a transient decrease in serum testosterone to undetectable levels at 8 and 10 weeks after the initial vaccination.

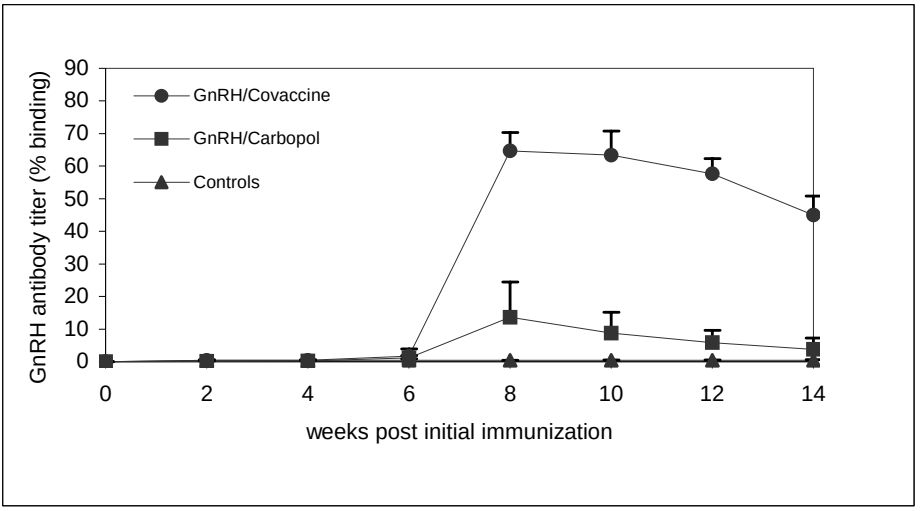

Figure 1. Mean antibody titers against GnRH measured with a radio-immuno-assay and presented as the percentage of iodinated GnRH bound by a 1:2000 final serum dilution of stallions injected, at day 0 and 6 weeks later, with the G6k-GnRH-tandem-dimer-OVA-conjugate in CoVaccine^{TM} HT adjuvant (GnRH/CoVaccine), or the G6k-GnRH-tandem-dimer-OVA conjugate in Carbopol adjuvant (GnRH/Carbopol) or with the control emulsion (Controls).

The deprivation of testosterone was reflected in the quality of the semen (Table 2). Within four weeks after the second vaccination, sperm motility was reduced in all four stallions of the GnRH/CoVaccine group (slope = -3.0 ± 1.3; p = 0.01), resulting in a lower 'post booster' sperm motility as in the control stallions (p=0.02). Stallion 794 of the GnRH/Carbopol group, also showed a decrease in sperm motility. No effect of immunization on semen volume, sperm concentration and sperm counts were observed for treatment groups, except that sperm concentration in the stallions of the GnRH/Carbopol vaccine was lower (p=0.03) as compared to the control stallions. However effects were seen for individual stallions (Table 2). Stallion 796, 802 and 811 of the GnRH/CoVaccine group and stallion 794 of the GnRH /Carbopol group showed a reduction in total sperm counts throughout the experiment (slope < -235). A distinct effect of GnRH immunization was seen in stallion 796 (GnRH/CoVaccine) and stallion 794 (GnRH/Carbopol). Both stallions did not ejaculate at 13 weeks after the first immunization due to a poor libido.
Testis size at the end of the experiment, week 14, was diminished in three stallions treated with GnRH/CoVaccine and one stallion (stallion 794) treated with GnRH/Carbopol (Figure 3). In these stallions the length of the longest axis of the testis ranged between 6 and 6.5 cm at the first and second immunization and decreased to 4 - 5 cm at 14 weeks after the initial immunization. Testicles were 23-33% smaller as compared to initial measurements.

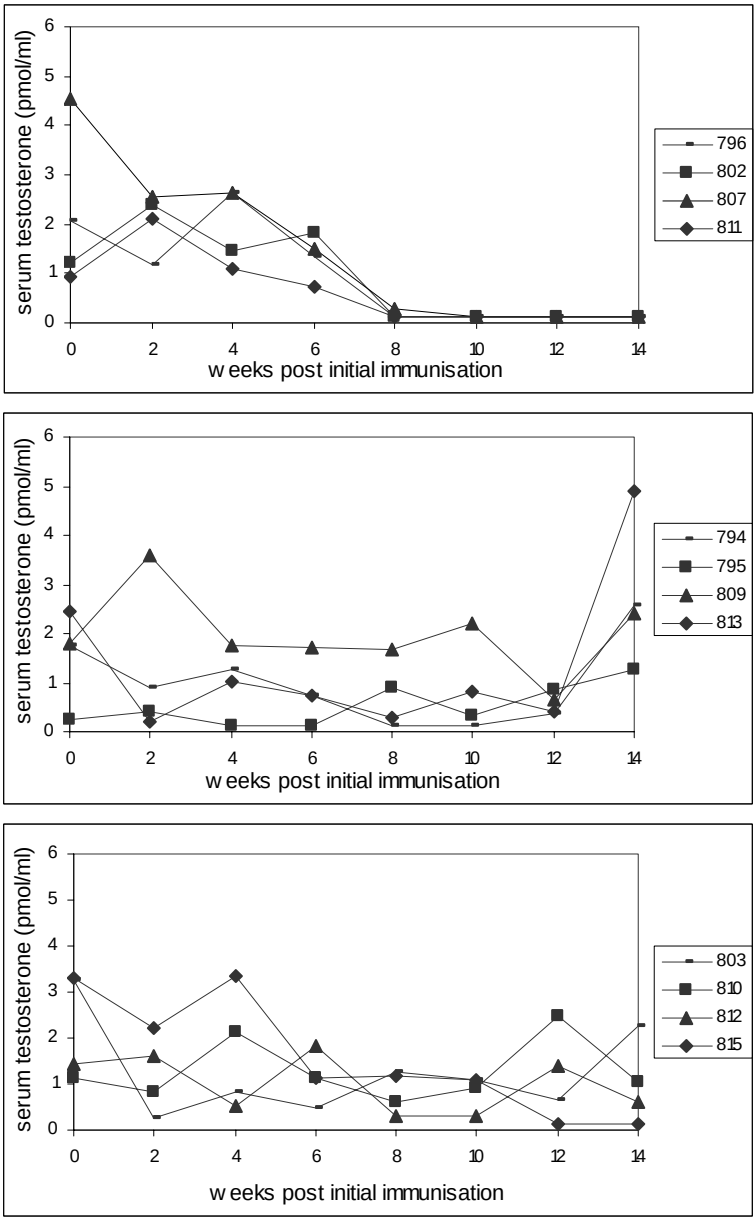

Figure 2. Serum testosterone concentrations of stallions injected, at day 0 and 6 weeks later, with GnRH/CoVaccine (upper panel), GnRH/Carbopol (middle panel) and with the control emulsion (lower panel).

Table 2. Sperm characteristics of pony stallions immunized at week 0 and 6 with G6k-GnRH-tandem-dimer-OVA conjugate in CoVaccine^{TM} HT adjuvant (GnRH/CoVaccine) or Carbopol (GnRH/Carbopol) and of control stallions. Values are expressed as the slope of values of week 0, 2, 4, 6, 8, 10 and 13 (slope) and as 'post booster values', the mean value of week 8, 10, 13 (week 8-13).

	Semen volume (ml)		Sperm motility (%)		Sperm concentration (x10^6 / ml)		Total number of sperm (x10^6)	
Treatment / stallion	slope	week 8-13	slope	Week 8-13	slope	week 8-13	slope	week 8-13
GnRH/CoVaccine								
796*	-1.9	12	-4.8	2	-8.1	143	-294	2389
802	-2.6	9	-2.3	57	5.4	243	-236	2155
807	-0.5	24	-1.7	47	1.2	185	-43	4468
811	-0.5	13	-3.1	35	-12.7	226	-334	2535
mean	-1.4	14.5	-3.0[a]	35.3[a]	-3.6	199	-227	2887
GnRH/Carbopol								
794*	-1.1	11	-4.4	23	-10.4	38	-244	536
795	0.2	14	0.7	72	-7.7	92	-88	1259
809	1.3	40	1.6	38	0.8	42	35	1056
813	-1.1	28	0.7	70	1.5	174	193	3912
mean	-0.2	23.3	-0.4	50.8	-4.0	87[a]	-26	1691
Controls								
803	0.0	8	0.3	65	-9.7	313	-107	2473
810	-0.6	20	0.5	78	2.1	176	-54	3603
812	0.3	25	0.5	57	-5.2	127	-43	3172
815	-0.2	29	0.6	83	-2.5	201	-97	5653
mean	-0.1	20.5	0.5[b]	70.8[b]	-3.8	204[b]	-75	3725

* values of week 13 are not included, because no ejaculate could be collected at that time due to suppressed libido

[a, b] Means within a column with a different superscript letter differ (P<0.05)

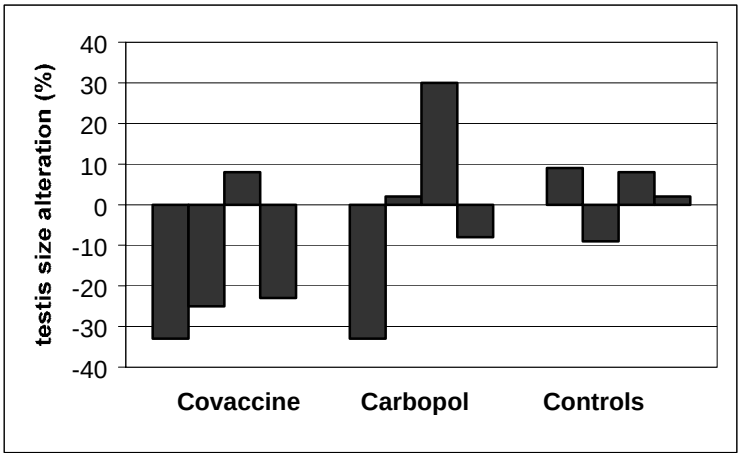

Figure 3. Alterations in mean testis lenght during the experimental period for individual stallions treated with GnRH/CoVaccine (nrs. 796, 802, 807, 811), GnRH/Carbopol (nrs.794, 795, 809, 813) and the control emulsion (nrs. 803, 810, 812, 815).

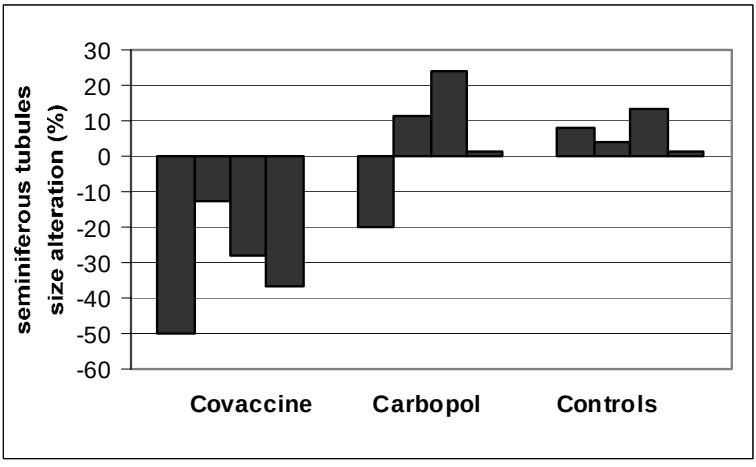

Figure 4. Alterations in mean seminiferous tubuli diameter during the experimental period for individual stallions treated with GnRH/CoVaccine (nrs. 796, 802, 807, 811), GnRH/Carbopol (nrs. 794, 795, 809, 813) and with the control emulsion (nrs. 803, 810, 812, 815).

Evaluation of the diameter of the seminiferous tubules demonstrated that the mean diameter at the end of the experiment was reduced by 10 to 50% as compared to the mean diameter prior to immunization in all four stallions treated with GnRH/CoVaccine and in stallion 794 treated with GnRH/Carbopol (Figure 4). Mean seminiferous tubules diameter in the GnRH/CoVaccine group was smaller as compared to the mean diameter of the control group ($p = 0.03$).
In order to quantify the changes in the seminiferous epithelium, a Johnson score analysis was carried out. The Johnson score was substantially decreased in all stallions of the GnRH/CoVaccine group and in two stallions (794 and 809) of the GnRH/Carbopol group compared to the control group (Figure 5). The mean Johnson score was significantly lower in both, the GnRH/CoVaccine and the GnRH/Carbopol group ($p = 0.01$).

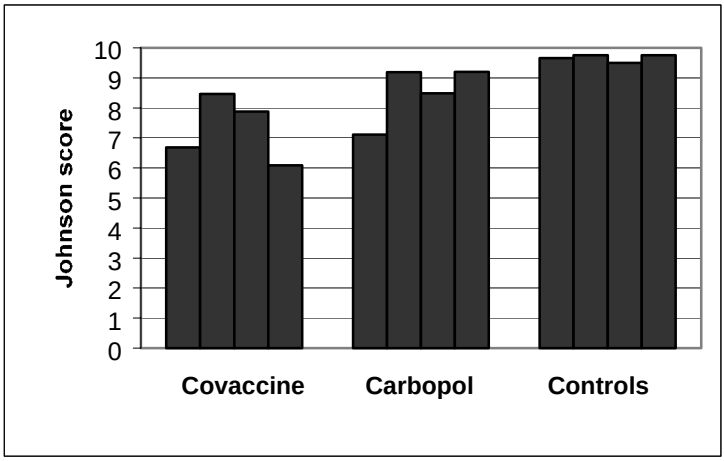

Figure 5. Johnson score for the individual stallions treated with GnRH/CoVaccine (nrs. 796, 802, 807, 811), GnRH/Carbopol (nrs.794, 795, 809, 813) and with the control emulsion (nrs. 803, 810, 812, 815).

Histological evaluation further showed that especially in the GnRH/CoVaccine group often the lumen in the seminiferous tubules was absent and spermatogenesis had become extremely disorganized (Figure 6). Generations of germ cells were sometimes missing resulting for instance in the observation that in the absence of round spermatids, elangating spermatids/spermatozoa and spermatocytes, were found directly next to each other. Besides the absence of round spermatids, spermatozoa were also regularly absent in the tubular cross-sections, resulting in a lower Johnson score. Though spermatogenesis was normal in most tubules in the GnRH/Carbopol group, except for horse 794, a considerable number of

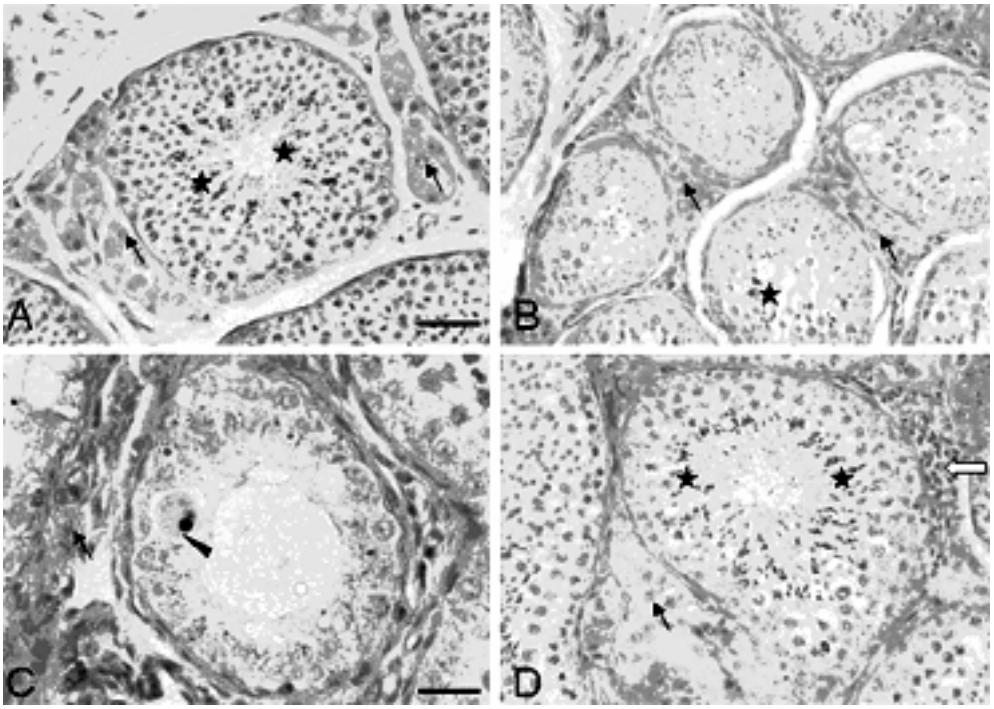

Figure 6. A) Cross section of the testis of stallion 803 (control). Spermatogenensis is normal, many layers of different generation of germ cells are present, such as elongating spermatids, as well as a tubular lumen. In the interstitium Leydig cells can be easily recognized by the presence of a large round nucleus and one or two nucleoli. B) Cross section of the testis of stallion 811 immunized with GnRH/CoVaccine. In the seminiferous elongated spermatids are absent or severely reduced in number. The Leydig cells have atrophied. C) Cross section of the same testis as shown in (B) at a higher magnification. Note the absence of elongated spermatids and the reduced size of the Leydig cells. D) Cross section of the testis of stallion 795 immunized with GnRH/Carbopol. Many seminiferous tubules show a normal morphology, Leydig cell morphology is identical to the controls. Local infiltration of immune cells is regularly observed. Elongated spermatids are indicated by asterisks, an apoptotic germ cell by an arrow head, Leydig cells by filled arrows, and infiltrating immune cells by an open arrow. The magnification of figures a, b and d are the same (original magnification x235, bar 54 μm), while the magnification of figure c is x475 (bar 27 μm).

tubules had no or a reduced lumen and spermatogenesis was slightly disorganized (Figure 6d). Unfortunately, this does not become apparent by the Johnson score analysis, since this method uses the presence or absence of specific germ cell types (table 1) as a measure to determine the score and not the location of the cells within the seminiferous epithelium. In the GnRH/Carbopol group signs of inflammation were regularly observed in the interstitial

compartment (Figure 6d) as well as sometimes in degenerating seminiferous tubules (stallion 794). In the GnRH/CoVaccine and the control group the incidence of inflammation was less when compared to the GnRH/Carbopol group. In the GnRH/Covaccine group the Leydig cells were atrophied in all animals, while in the GnRH/Carbopol group this was only apparent in animal 794. In the other 3 animals of this group Leydig cell morphology was more or less normal (Figure 6d) and similar to Leydig cells of control stallions (Figure 6a).

4. DISCUSSION

In the present study we have demonstrated full efficacy of a GnRH vaccine in sexually mature stallions, without the occurrence of adverse reactions due to vaccination. In other studies GnRH immunization has proven to be successful in yearling and two-year old stallions after vaccination with a GnRH vaccine based on mineral oil and a water-soluble vaccine (Dowsett et al. 1991, 1993, 1996). The oil-based vaccine was not well tolerated since severe local reactions appeared at the injection site. Only recently, a GnRH vaccine was tested in sexually mature stallions (Malmgren et al. 2001). It appeared to be effective in 2 out of 3 treated stallions, though 5 injections were required and adverse reactions at the site of injection were observed, despite the fact that a mild adjuvant (Equimune and aluminium hydroxide) was used. As mineral oil adjuvants induced severe injection site lesions (Dowsett et al., 1991), we selected adjuvants without mineral oil. After injection with these vaccines, no tissue reactions or behavioural changes were observed in any of the stallions, demonstrating that both adjuvants are suitable for practical use in horses.
After the first immunization, no significant antibody responses could be detected at the given serum dilution in the stallions of both GnRH vaccinated groups. However, within two weeks after the booster immunization, high antibody titers were present in all animals of the GnRH/CoVaccine group. This subsequently resulted in depression of testosterone concentrations, in all stallions treated with this vaccine two weeks after the second immunization. These results indicate that with the GnRH/CoVaccine vaccine, the time of onset of 'castration' can be regulated by the timing of the booster vaccination: before the booster vaccination only minor effects of vaccination could be observed, while 'castration' effects were evident shortly after booster vaccination in all treated stallions.
In contrast to the high and consistent antibody response in the GnRH/CoVaccine group after the second immunization, antibody responses in the GnRH/Carbopol treated stallions exhibited a large variation (1-27%). Titers were only slightly increased in two stallions, while two other stallions responded with substantial antibody titers. Such a large variation in antibody responses after GnRH immunization is shown in the majority of the GnRH-immunization studies in which sexually mature subjects are involved (Chappel et al., 1980,

Lincoln et al., 1982, Schanbacher et al., 1983, Grizzle et al., 1987, Simms et al., 2000, Malmgren et al., 2001). Several factors may affect the immunogenicity of the applied vaccines, such as the type and amount of antigen, the adjuvant, vaccination schedule and the species involved. In the present study it is evident that CoVaccine^{TM} HT adjuvant enhances immunogenicity of the antigen more than Carbopol adjuvant.
Antibody titers of the two responding stallions of the GnRH/Carbopol group were much lower than titers of the GnRH/CoVaccine treated stallions and only caused a temporary reduction of serum testosterone in one of the stallions (794). Suprisingly, this transient suppression of testosterone resulted in dramatic effects on sperm motility and a loss of libido, which was also seen in one stallion of the GnRH/CoVaccine group. In the three remaining stallions of the GnRH/CoVaccine group, which exhibited much higher antibody titers and undetectable testosterone concentrations for a longer period then stallion 794 of the GnRH/Carbopol group, effects on sperm motility and libido were less. This may indicate that additional effects such as infiltration of immune cells as was observed in stallion 794 may also have affected testis morphology and sperm characteristics.
We believe that the effects of GnRH-immunization on sperm characteristics in the stallions treated with GnRH/CoVaccine may become more apparent when at least one spermatogenic cycle is completed in the presence of prolonged depression of serum testosterone levels. In this study testosterone levels were suppressed during the last 7 weeks of the experiment, which is less then time neccessary to complete one spermatogenic cycle, i.e. 8 weeks. Althought effects on sperm production were not yet apparent, testosterone suppression did affect sperm motility indicating that testosterone deficiency is affecting sperm maturation (i.e. potential for motility), in the epididymus.
Only one of the two stallions of the GnRH/Carbopol group, which raised substantial antibody titers (stallion 794), exhibited effects on testosterone concentrations, whereas in the other responding stallion of the GnRH /Carbopol group no effects were seen, despite similar antibody titers. This indicates that intermediate antibody titers may lead to 'castration' effects in some, but not all animals (Zhang et al., 1999, Turkstra et al., 2002).
Testis size was reduced to the same extent as reported in other GnRH immunization studies in stallions (Dowsett et al. 1991, 1993, 1996, Malmgren et al., 2001). However, in the studies of Dowsett, relatively young stallions were involved of which the testes most likely were still developing. This probably enlarged the difference between treated and control stallions.
Testis histology was affected in all stallions treated with GnRH/CoVaccine and in stallion 794 treated with GnRH/Carbopol group as indicated by a substantial decrease in tubuli diameter and affected spermatogenesis. These results are in line with the observed reduction of the testis size, although in stallion 807 of the GnRH/CoVaccine group, testis size was not reduced when compared to the start of the experiment. However, the reduced

diameter of the tubules, the absence of the tubules lumen and the reduced numbers of tubules with elongating spermatids and spermatozoa, clearly indicates the severe effects on spermatogenesis in this and all other stallions of the GnRH/CoVaccine group. Presumably, the effects on spermatogenesis would have been more severe when the experiment had been extended for more than 15 weeks.

5. CONCLUSION

In this study, two immunizations with G6k-GnRH-tandem-dimer OVA conjugate in a suitable adjuvant, such as CoVaccine^{TM} HT causes a rapid and maximum reduction in testosterone levels in sexually mature stallions, subsequently causing reduced sperm motility and affected testis function, while no adverse reactions after immunizations were observed.

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Chapter 8

Chronic toxicity and testosterone inhibitory effects of two potential GnRH human prostate cancer vaccines in young male pigs

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ABSTRACT
Active immunization against gonadotropin releasing hormone (GnRH) can be an alternative for hormone therapy in prostate cancer patients. Here we report a preclinical immunization study in young male pigs with the G6k-GnRH-tandem-dimer peptide conjugated to Keyhole Limpet Hemocyanin (KLH) and formulated with Montanide ISA 51 adjuvant (ISA 51) or CoVaccine HT adjuvant (CoVaccine).
Cross-bred male pigs (n=120) aged between 12 and 14 weeks were assigned to 8 treatments: surgical castrates, PBS controls, G6k-GnRH-tandem-dimer-KLH conjugate in ISA 51 (0, 400 or 800 µg conjugate) and in CoVaccine (0, 400 or 800 µg conjugate). Immunizations were given at week 0, 2 and 4. At week 12, 20 and 30 after initial immunization, 5 animals of each treatment group were sacrificed.
Immunization with 400 µg and 800 µg G6k-GnRH-tandem-dimer-KLH conjugate in CoVaccine resulted in undetectable plasma testosterone levels in all treated pigs from week 8 until the end of the study. Pigs treated with the conjugate in ISA 51 showed a variable response; serum testosterone levels were affected in some but not all animals. Testosterone depletion was accompanied by impaired testes growth, reduced relative weights of testes and accessory sex organs, and reduced sperm numbers and sperm motility.
Minor transient injection site reactions were observed after the second and third immunization in pigs treated with CoVaccine (0, 400 and 800 µg). Autopsy revealed that mainly mild injection site lesions were noted in CoVaccine treated animals, while moderate lesions were detected in pigs treated with ISA 51. Body weight and weight of heart, kidneys, liver, adrenals, spleen, thymus, brain and pituitary were not affected by GnRH immunization and macroscopic changes were not found in these organs or other non-reproductive tissues. Analysis of hematological, clinical biochemistry and urinalysis parameters revealed no remarkable changes, except increased plasma urea concentrations in the pigs immunized with the conjugate in CoVaccine adjuvant and the surgical castrates, suggesting that this may be due to testosterone depletion.
We conclude that immunization with the G6k-GnRH-tandem-dimer-KLH conjugate can cause a rapid reduction in serum testosterone, in particular in combination with CoVaccine adjuvant, while no chronic toxic effects were observed.

1. INTRODUCTION

Immunization against GnRH to reduce gonadal steroid secretion has been studied for various purposes. In animals it has been tested to improve growth performance, meat quality and handling of the animals, and to prevent pregnancies and undesired sexual behavior (Ladd et al., 1994; Bonneau and Enright, 1995; Millar et al., 2000; Robbins et al., 2004). In humans, its potential for treatment of endocrine disorders (Gual et al., 1997) and gonadal steroid-dependent tumor growth (Talwar et al., 1995; Simms et al., 2000) has been investigated.

In previous studies we used the G6k-GnRH-tandem dimer peptide conjugated to ovalbumin (OVA). For application in humans, antibodies raised against OVA may interact with normal food ingredients and induce undesired side effects. Therefore, to apply the conjugate in humans, a protein of non-mammalian origin was selected, namely keyhole limpet hemocyanin (KLH). KLH has been approved for clinical use in humans (Holmberg and Sandmaier, 2001; Naylor et al., 1991).

In order to initiate clinical trials in prostate cancer patients, a chronic toxicity study was performed in pigs. Two doses of the G6k-GnRH-tandem-dimer-KLH conjugate in two distinct adjuvants, namely Montanide ISA 51, a water-in-mineral oil adjuvant which is well tolerated by humans in clinical trials (Aucouturier et al., 2002), and the newly developed aqueous adjuvant CoVaccine HT (Blom and Hilgers, 2004) were evaluated. Besides local and systemic toxicity, effects on the hypothalamus-pituitary-gonadal axis were investigated.

2. MATERIALS AND METHODS

2.1 Animals

The experimental protocol describing the management, surgical procedures and animal care, was reviewed and approved by the Local Committee on Animal Care and Ethics. Hundred-twenty 12 to 14-week-old male commercial crossbred pigs were allocated to 8 treatment groups (group 1-8), each containing 3 subgroups of 5 pigs (subgroup A, B and C), using a random assignment procedure based on body mass (PROC PLAN, SAS Institute Inc., Cary, NC, USA, Release 8.2). Animals were kept in a controlled environment with a temperature of 21 ± 4°C, a relative humidity of 30-70% and a 12 hour light/12 hour dark cycle. Five animals of one subgroup were housed together in cages (140 x 330 cm) with slatted floors. From week 20 onwards, animals were housed with 2 or 3 males per cage. Animals had free access to standard pelleted pig feed and water.

2.2 Vaccines

2.2.1 Antigen

Synthesis of the G6k-GnRH-tandem peptide was performed on gram scale, by hand in a glass vessel using Fmoc-amino acids and FastMoc chemistry. The peptide sequence of the G6k-GnRH-tandem peptide in single letter amino acid code is: pEHWSYkLRPGQHWSYkLRPGC#, in which pE = pyroglutamic acid; # = amide and k = D-Lysine. The crude peptide was purified and subsequently dimerized by dissolving it in 20% DMSO in water. The dimerized peptide was purified and lyophilized for further processing. For conjugation of the peptide via N-ethyl-N-(3'-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) to KLH (Vacmune liquid, GMP grade, Biosyn, Germany), an equal weight of peptide and carrier protein was dissolved separately in water. The peptide solution was slowly added to the KLH solution under continuous stirring. Next a 10-fold excess, based on weight equivalents, of EDC was dissolved in water and slowly added to the solution of peptide/KLH. After at least 6 h slowly shaking, the product was dialyzed, lyophilized and stored at –20°C until immunization. One mg KLH was estimated to contain approximately 0.5 mg peptide as determined by comparative amino acid analysis.

2.2.2 Adjuvants and vaccine formulation

Two adjuvants were included in this study. CoVaccine HT (40 mg sucrose ester/ml) kindly provided by CoVaccine BV (Utrecht, The Netherlands) and Montanide ISA 51 (Seppic, Paris, France). One day prior to immunization, the conjugate was dissolved in water to which an equal volume of phosphate buffered saline (PBS) was added. The CoVaccine adjuvanted vaccines were formulated by mixing the antigen solution with one volume of adjuvant. The ISA 51 formulations were emulsified by repeatedly forcing a mixture of antigen solution and adjuvant (1:1) through a needle (18G) until an emulsion was obtained. Emulsions were stored overnight at 4°C and aspirated into 2 ml syringes prior to injection. Two ml of each emulsion was stored at 4°C for stability control. None of the emulsions showed separation of phases at day 1, day 8 or day 15.

2.3 Experimental design

Eight groups of 15 pigs received the following treatment:
group 1: Water / PBS (1:1) (PBS controls)
group 2: ISA 51 emulsion without G6k-GnRH-tandem-dimer-KLH conjugate (ISA-0)
group 3: ISA 51 emulsion containing 400 µg G6k-GnRH-tandem-dimer-KLH conjugate (ISA-400)
group 4: ISA 51 emulsion containing 800 µg G6k-GnRH-tandem-dimer-KLH conjugate (ISA-800)

group 5: CoVaccine formulation without G6k-GnRH-tandem-dimer-KLH conjugate (CoVaccine-0)
group 6: CoVaccine formulation containing 400 µg G6k-GnRH-tandem-dimer-KLH conjugate (CoVaccine-400)
group 7: CoVaccine formulation containing 800 µg G6k-GnRH-tandem-dimer-KLH conjugate (CoVaccine-800)
group 8: Surgical castrates

Pigs were immunized at days 0, 14 and 27, in the left side of the neck and in the musculus semitendinosus of the left and right hind leg, respectively. Two ml of the vaccines was injected intramuscularly. The sites of injection were inspected for adverse reactions according to a fixed scale, one day prior to each administration, two hours after administration and 2, 4, 6 and 10 days after administration. The rectal temperature of all animals in subgroup C of each treatment group was measured one day prior to immunization and 2 hours, 2, 4 and 6 days after injection. Clinical observations were made twice daily. Males of group 8 were castrated by surgical removal of the testes on day 28 of the study.
Of each treatment group (1-8), animals of subgroups A, B and C were sacrificed at 12, 20 and 30 weeks after initial immunization, respectively.

2.4 Sample collection
Blood samples for hematological and clinical biochemical analysis and antibody and hormone determinations were collected from all animals 1 day prior to the first immunization and at week 4, 8, 12, 20 and 30 after initial immunization. Blood samples were drawn from the jugular vein. Blood was collected in uncoated serum tubes, tubes coated with EDTA and tubes coated with Li-heparin. Urine samples were collected with a cup from naturally urinating animals of the subgroups C, before the start of the study and at week 4, 8, 12, 20 and 30 after the first immunization.

2.5 Assays and measurements
2.5.1 Testis size
Changes in testis size were measured at fortnightly intervals by measuring testis length using a vernier caliper. The pre-treatment measurement revealed that there were differences among treatment (sub)groups, therefore, the subsequent measurements were calculated as percent of the mean pre-treatment value per subgroup. A reduction of 100% means that testes were not palpable.

2.5.2 GnRH antibody titer

GnRH specific antibodies were measured with an ELISA as described previously (Turkstra et al., 2002) with the following adaptations. An extended GnRH peptide (GnRH-Gly-Gly-Lys) was coated overnight and the next day serial serum dilutions were allowed to react with the peptide for 1 hour at 37°C. The antibody titer was calculated as the log of the dilution factor that gave an optical density 4 times higher than the background value.

2.5.3 Plasma testosterone

Plasma testosterone concentrations were determined in duplicate with a commercially available radioimmunoassay (DPC, Breda, The Netherlands) according to the instructions of the manufacturer. The intra- and interassay variability were below 5.6% and 7.1% respectively. The detection limit of the assay was 0.04 ng/ml.

2.5.4 Plasma gonadotropin levels

The concentrations of LH and FSH were measured in duplicate in plasma by the double-antibody competitive RIA method (Van de Wiel et al., 1981) using the RIA kits supplied by the National Hormone and Peptide Program (NHPP) from the National Institute of Diabetes and Digestive and Kidney diseases (NIDDK) and Dr. A.F. Parlow. The LH antibody (NIDDK: AFP-15103194) was used in a final dilution of 4.48×10^{-6} and the LH standards were prepared from NIDDK; AFP-11043B. The detection limit of the assay was 0.143 ng/ml. The average intra-assay and inter-assay coefficient of variation of two control samples were 4.4% and 5.9%, respectively. The standards for the FSH RIA were prepared from NIDDK-AFP-10640B and the FSH antibody (NIDDK-AFP-2062096) was used in a final dilution of 4.48×10^{-5}. The assay had a detection limit of 0.373 ng/ml, and an average intra-assay and inter-assay coefficients of variation of 9.0% and 12.3%, respectively.

2.6 Sperm number and sperm quality

At autopsy, from each animal the right cauda epididymis was minced, washed and the sperm concentration of the obtained fluid was determined turbidimetrically. Sperm motility and the percentage of live sperm cells were determined according to Woelders et al. (1997) with the exception that sperm motility was determined using phase contrast microscopy. Acrosome intactness (normal apical ridge) was determined as described by Pursel et al. (1972). The percentage of sperm with a normal morphology was determined in dried smears of semen mixed in half a volume of an eosin/aniline solution in water (eosin 4 g/l, aniline 64 g/l). Cells were classified as abnormal when they had an abnormally shaped acrosome or head, a loose head (no tail), a malformed tail, or a protoplasmic droplet in the neck region. Sperm cells with a more distal or with no protoplasmic droplet were considered morphologically normal.

2.7 Hematological, clinical biochemical and urine analysis

Hematological, clinical biochemical and urine analysis were carried out in the Chemical and Endocrinology Laboratory of Animal Sciences Group (Lelystad, The Netherlands) using internal standard operating procedures according to the OECD principles of Good Laboratory Practice (Directive 87/18/EEC, 88/320/EEC). The following parameters were determined in blood: white blood cell, red blood cell, platelet, granulocyte, lymphocyte, monocyte, eosinophil, basophil and myelocyte counts, concentrations of hemoglobin, hematocrit, mean corpuscular hemoglobin and mean corpuscular volume. In plasma samples, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, total bilirubin, glucose, creatinine, urea, total protein, protein albumin, protein globulin, albumin globulin ratio, total cholesterol, triglycerides, non-esterified fatty acids, sodium, potassium, chloride, calcium and phosphorus concentrations were determined. The following urinalysis parameters were determined: colour, clarity, pH, as well as protein, glucose, ketones, bilirubin, blood, urobilinogen, nitrite, ascorbic acid, leucocytes, sodium, potassium and calcium concentrations.

2.8 Autopsy

One day prior to sacrifice, animals were weighed, while feed was withheld the morning before the animals were sacrificed. Animals were electrocuted, subsequently exsanguinated and dissected. Macroscopical abnormalities were recorded. The following organ weights were recorded: adrenal glands, brain, bulbourethral glands, epididymides, heart, kidneys, liver, pituitary, prostate, seminal vesicles including coagulating gland and fluids, spleen, testes and thymus. Injection sites were fixed, embedded in paraffin, cut at a thickness of 2-4 micrometers and stained with hematoxylin and eosin for histopathological analysis.

2.9 Statistical analysis

For parametric variables, an analysis of variance (ANOVA) was performed with 8 treatment groups (PBS control, ISA-0, ISA-400, ISA-800, CoVaccine-0, CoVaccine-400, CoVaccine-800 and surgical castrates) and 3 sub groups (A,B,C) before treatment was started in order to address the question whether there were a priori group differences despite the random matched assignment of animals based on body weights. Next, using one-factorial ANOVA with 8 treatment groups, a comparison of pre-treatment values was performed, and treatment effects were analyzed on the data of weeks 12, 20 and 30 of the study. In each ANOVA, the measurements of all animals per treatment were included, except for urinalysis, which included only the 5 pigs of subgroup C of each treatment group. Some urinalysis parameters were not statistically analyzed, due to the non-parametric distribution of the data and the low number of animals per treatment group. Sperm data and organ weights of all subgroups within one treatment were combined and

treatment effects were analyzed by ANOVA. Because of differences in body weight of the subgroups at the time of sacrifice (week 12, 20 or 30), all organ weights were expressed as percentage of body weight. All ANOVAs were supplemented with Sidak post-hoc pair wise comparisons ($p=0.05$) between PBS controls and other treatment groups for each time point

3. RESULTS

3.1 Vaccine efficacy

As shown in figure 1, antibody titers increased after immunization and were already at a high level at week 4 in CoVaccine-400 and CoVaccine-800 treated animals. All GnRH immunized groups reached maximum antibody titers at week 4 or week 8. Thereafter, titers slowly decreased and stayed at a constant level at week 20 and 30. No differences between GnRH immunized groups were determined at week 12, 20 or 30.

Plasma LH and FSH concentrations of the surgical castrated pigs were elevated after castration and were significantly higher than for PBS controls ($p<0.05$). In general, GnRH immunization lowered FSH levels, but these differences were not confirmed statistically (Figure 2), As shown in figure 3, LH levels declined after GnRH immunization, but were only significantly lower than those of the PBS controls ($p<0.05$) in the CoVaccine-800 group at week 12 and 20.

Castrate-like testosterone profiles were established in all pigs treated with CoVaccine-400 and CoVaccine-800 (Figure 4); testosterone concentrations in plasma were below the detection limit of the assay from week 8 till the end of the study. As a consequence, testosterone levels of CoVaccine-400 and CoVaccine-800 groups and the surgical castrates were lower ($p<0.05$) than those of the PBS controls at week 12 and 20. The ISA-400 and ISA-800 groups had intermediate testosterone levels.

Testis size measurements at week 12 revealed that the testes of GnRH immunized pigs were significantly smaller ($p<0.05$) than those of the PBS controls (Figure 5). The pigs in the CoVaccine-400 and CoVaccine-800 group showed a larger reduction in testes size than the pigs in the ISA-400 and ISA-800 group. Effects of the CoVaccine-400 and CoVaccine-800 treatment on testis growth was even more pronounced at week 20 and 30; in the majority of the pigs testes were not palpable.

Testes and accessory sex gland weight (Table 1) were significantly reduced ($p<0.05$) in pigs treated with ISA-800, CoVaccine-400 and CoVaccine-800 and in surgical castrates as compared to the PBS control group, whereas in the ISA-400 group only the testes weighed less than those of the PBS controls ($p<0.05$).

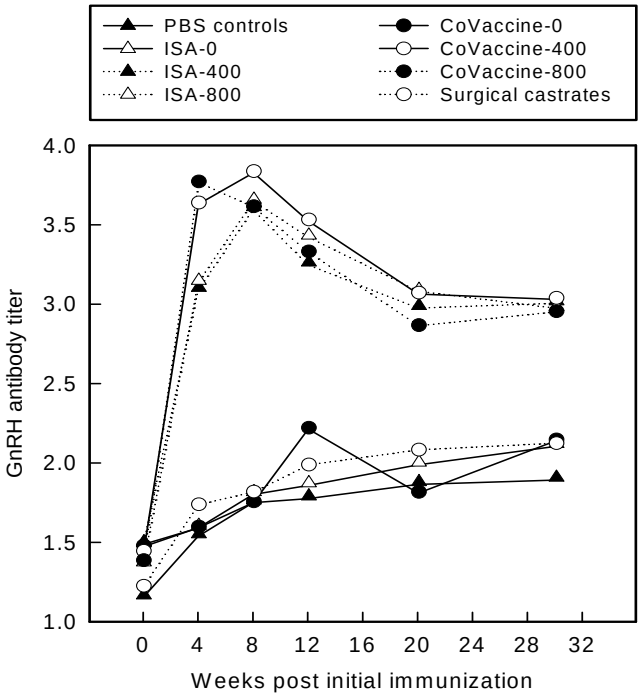

Figure 1. GnRH antibody titers in plasma of pigs treated with formulations as indicated above the figure. Immunizations were given at week 0, 2 and 4. Each point represents the mean titer of all animals per treatment group as a function of time after initial immunization.

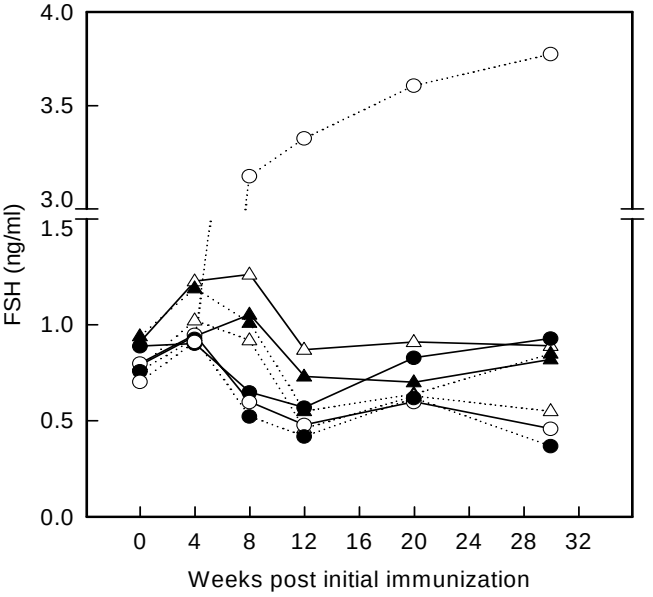

Figure 2. FSH concentrations in plasma of pigs treated with formulations as indicated in figure 1. Immunizations were given at week 0, 2 and 4. Each point represents the mean FSH concentration of all animals per treatment group as a function of time after initial immunization.

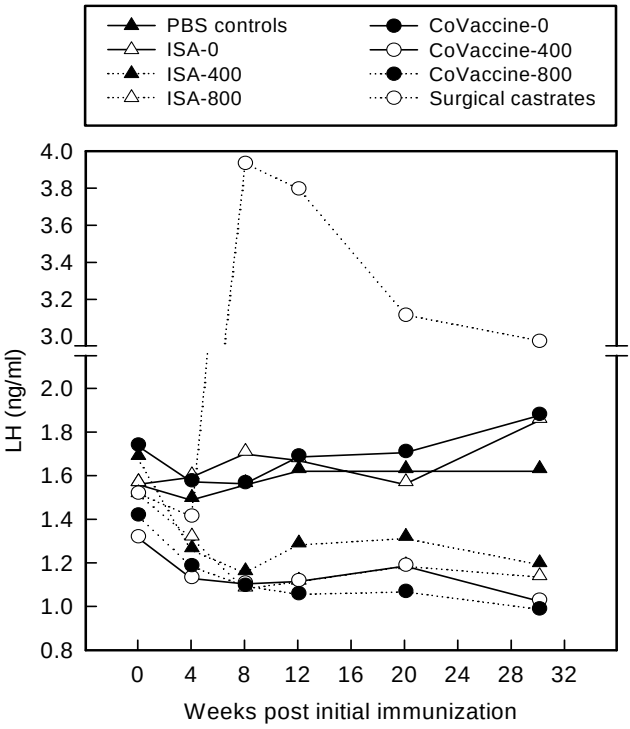

Figure 3. LH concentrations in plasma of pigs treated with formulations as indicated above the figure. Immunizations were given at week 0, 2 and 4. Each point represents the mean LH concentration of all animals per treatment group as a function of time after initial immunization.

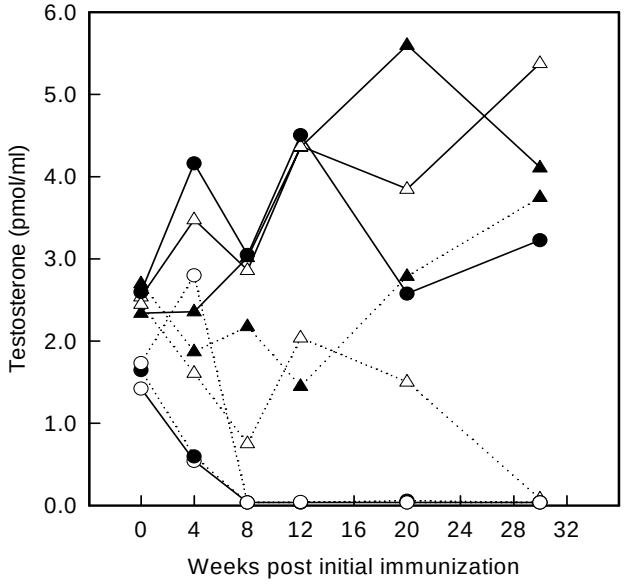

Figure 4. Testosterone concentrations in plasma of pigs treated with formulations as indicated in figure 3. Immunizations were given at week 0, 2 and 4. Each point represents the mean testosterone concentration of all animals per treatment group as a function of time after initial immunization.

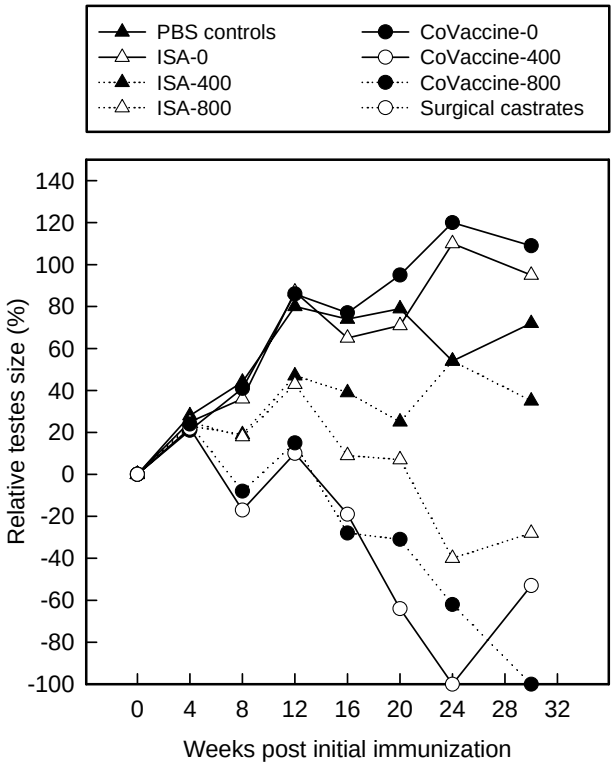

Figure 5. Testis length in pigs treated at week 0, 2 and 4, with formulations as indicated above the figure. Each point represents the mean relative testis length as compared to the pre-immunization values. A reduction of 100% means that testes were not palpable.

The total number of sperm in the cauda epididymis was reduced in all GnRH immunized groups ($p<0.05$; Table 2). In 10 out of 28 pigs of the CoVaccine-400 and CoVaccine-800 group no sperm was detectable, while in the remaining pigs sperm numbers ranged from 10^6 to $2.19x10^9$ sperm cells. In 3 out of 30 pigs of the ISA-400 and ISA-800 groups, no sperm could be detected, in 14 animals sperm numbers ranged from 10^6 to $4.25x10^9$ sperm cells and in the remaining animals, sperm counts were normal. Sperm quality as determined by % motile sperm, % sperm with normal apical ridge, % normal sperm and % live sperm was decreased in the CoVaccine-400 and CoVaccine-800 group ($p<0.05$). In the ISA-400 and ISA-800 group sperm quality was only slightly reduced (Table 2).

Table 1: Effect of GnRH immunization on the weight ratios of testis and accessory sex glands.

Measure	PBS controls	ISA-0	ISA-400	ISA-800	CoVaccine-0	CoVaccine-400	CoVaccine-800	Surgical castrates
Testis (g)	1.76±0.07	1.86±0.11	1.18±0.19*	0.88±0.17*	2.04±0.13	0.26±0.02*	0.38±0.05*	-
Epididymis (g)	0.53±0.05	0.51±0.05	0.36±0.05	0.30±0.07*	0.55±0.05	0.12±0.02*	0.14±0.02*	-
Bulbourethral gl. (g)	0.63±0.05	0.78±0.08	0.43±0.09	0.26±0.05*	0.73±0.09	0.10±0.01*	0.13±0.02*	0.12±0.01*
Prostate (mg)	42±4	45±5	32±7	15±2*	49±1	8±1*	10±1*	9±2*
Seminal Vesicle (g)	1.72±0.11	2.15±0.31	1.06±0.37	0.54±0.19*	1.90±0.29	0.08±0.01*	0.10±0.02*	0.10±0.01*

Weight ratios are expressed as mean ±SE weight of organs per kg body weight; * Means within a row differ from PBS controls ($p<0.05$) n=15, except for CoVaccine-0, CoVaccine-400 and CoVaccine-800 (n=14) and surgical castrates (n=13); For testis, epididymis and bulbourethral gland only the weights of the right organs are listed.

Table 2: Mean sperm number and sperm quality per treatment group[a].

Measure[b]	PBS controls	ISA-0	ISA-400	ISA-800	CoVaccine-0	CoVaccine-400	CoVaccine-800
Total sperm number ($x10^9$)	27.401	26.765	14.780*	8.276*	27.112 (14)	0.008* (14)	0.377* (14)
% Motile sperm	64	71 (14)	51	54 (12)	72 (13)	15* (5)	30* (8)
% Sperm with NAR[c]	89	93 (14)	60*	68 (12)	94 (13)	16* (7)	36* (8)
% Normal sperm	85	88	60*	47* (12)	87 (13)	13* (7)	8* (8)
% Live sperm	89	87	62*	68 (12)	89 (13)	26* (7)	45* (8)

[a] n=15, unless indicated otherwise between brackets; [b] Determination of sperm number and sperm quality is described in Materials and Methods; [c] NAR normal apical ridge;
* Significantly reduced compared to PBS controls ($p<0.05$)

Overall, the number of sperm cells was related to the testis weight. Sperm counts were less than 5 x 10^9 in all animals with testis weighing less than 110 g, while in all animals, except one, with testes weighing more than 200 g, sperm counts were higher than 10 x 10^9 (Figure 6a). Sperm quality was related to testis weight and sperm output. Sperm quality was normal in animals with testis weighing more than 200 g and/or sperm counts higher than $5x10^9$ (Figure 6b-d). Below these thresholds, sperm quality varied, but appeared to be positively related to the total sperm number.

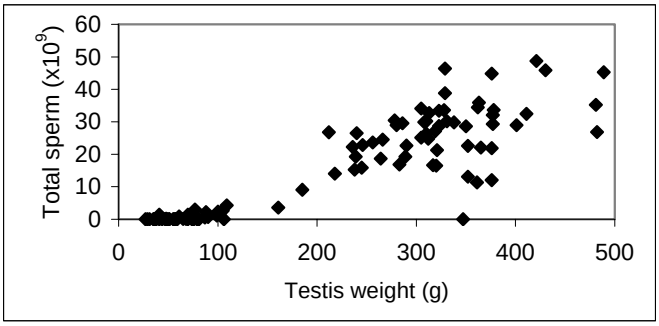

Figure 6a. Relation between total sperm number and mean testis weight of individual animals. Sperm was obtained from the right epididymis at autopsy (n = 102).

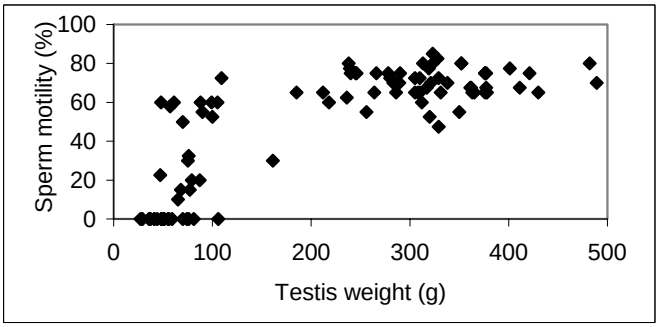

Figure 6b. Relation between sperm motility expressed as percentage motile sperm of the total sperm number and mean testis weight of individual animals. Sperm was obtained from the right epididymis at autopsy (n = 82).

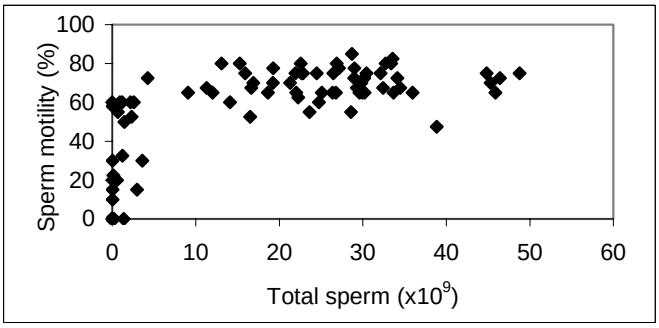

Figure 6c. Relation between sperm motility expressed as percentage motile sperm of the total sperm number and total sperm number of individual animals. Sperm was obtained from the right epididymis at autopsy (n = 82).

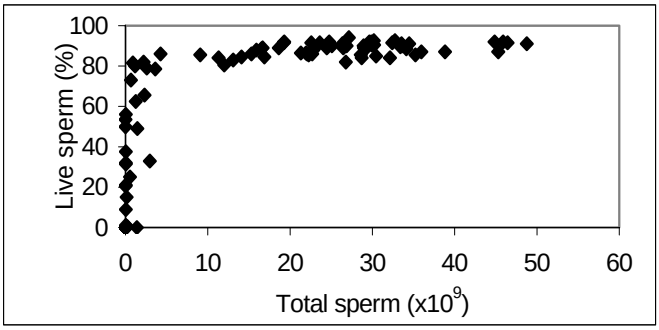

Figure 6d. Relation between percentage live sperm of total sperm number and total sperm number of individual animals. Sperm was obtained from the right epididymis at autopsy (n = 85).

3.2 Chronic toxicity

No effects of the treatments on body weight were detected when compared to the PBS controls. The mean body weight at 1 week prior to the start of the study was 64.0±6.4 kg (Mean ±SE) and at week 12, 20 and 30, mean body weights had increased to 141.5±13.9, 180.7±20.2, 213.9±24.4 kg, respectively.

During the study, 5 animals died or were euthanized due to reasons not related to the treatment. These animals were excluded from the autopsy data. No significant differences between PBS controls and other treatments were noted for organ weights (Table 3). At autopsy no treatment related macroscopic abnormalities in organs or tissues were observed. Biochemical analysis showed a significantly higher ($p<0.05$) urea concentration in plasma of surgical castrates and CoVaccine-400 and CoVaccine-800 treated animals compared to the PBS control group at week 12, while levels of the ISA-400 and ISA-800 group appeared to be higher than those of the PBS controls (Table 4). Moreover, increased urea concentrations for these 5 treatment groups were observed in week 8, 20 and 30. However, none of these apparent increases was confirmed statistically. Changes for other parameters were transient. Bilirubine levels were below the detection limit of the assay.

Pair-wise comparisons between PBS controls and other treatment groups for hematological parameters revealed no consistent pattern of treatment effects. Observed differences appeared to be transient and occurred at only one time point (data not shown).

Urinalysis revealed no abnormal values (data not shown)

Table 3: Effects of GnRH immunization on organ weight ratios.

Measure	PBS controls	ISA-0	ISA-400	ISA-800	CoVaccine-0	CoVaccine-400	CoVaccine-800	Surgical castrates
Heart (g)	2.92 ± 0.09	2.91 ± 0.08	2.92 ± 0.10	2.77±0.05	3.13±0.13	2.87±0.08	2.80±0.07	2.86±0.09
Liver (g)	12.82±0.90	12.79±0.59	11.52±0.58	11.44±0.43	11.91±0.54	11.65±0.40	11.63±0.44	11.12±0.56
Kidney (g)	1.34±0.05	1.39±0.06	1.21±0.05	1.20±0.08	1.27±0.07	1.17±0.05	1.17±0.07	1.19±0.10
Adrenal (mg)	16.5±0.7	17.4±1.3	17.0±0.8	18.3±1.5	15.3±1.1	16.4±1.1	16.6±1.2	16.4±1.9
Spleen (g)	1.91±0.35	1.56±0.08	1.36±0.07	1.42±0.08	1.53±0.12	1.72±0.32	1.46±0.07	1.43±0.20
Thymus (g)	0.41±0.04	0.36±0.04	0.36±0.40	0.48±0.04	0.39±0.04	0.42±0.05	0.41±0.04	0.39±0.04
Brain (g)	0.63±0.03	0.65±0.03	0.72±0.03	0.66±0.03	0.69±0.05	0.66±0.03	0.65±0.04	0.68±0.04
Pituitary	1.77±0.21	1.79±0.20	2.12±0.15	1.92±0.17	2.01±0.15	1.75±0.24	1.52±0.16	1.60±0.16

Values are expressed as mean ±SE; n=15, except for CoVaccine-0, CoVaccine-400 and CoVaccine-800 (n=14) and surgical castrates (n=13);
For kidney and adrenal only weights of the right organs are listed

Table 4: Effects of GnRH immunization on clinical biochemical values in plasma at week 12

	PBS controls	ISA-O	ISA-400	ISA-800	CoVaccine-0	CoVaccine-400	CoVaccine-800	Surgical castrates
Aspartate amino-transferase, U/L	43.0±13.2	30.9±2.0	28.8±1.8	28.5±1.5	30.1±1.7	26.6±0.7	30.5±.2	26.1±1.3
Alanine amino-transferase U/L	41.1±1.9	38.6±2.1	40.5±1.7	44.8±2.0	39.2±2.0	43.3±1.6	45.5±1.4	39.3±1.9
Alkaline phos-phatase U/L	94.1±5.1	85.3±8.8	85.1±8.7	85.4±9.0	96.9±3.9	91.3±5.1	75.9±7.0	81.8±7.3
Glucose, mM	4.15±0.18	4.94±0.18	4.86±0.13	4.98±0.15	5.22±0.14	4.92±0.12	5.16±0.22	5.20±0.1
Creatinine, µM	180.3±3.3	179.4±4.5	172.8±4.2	174.5±5.4	156.6±3.3*	158.5±3.3	159.6±6.9	159.4±7.0
Urea, mM	3.87±0.21	3.56±0.3	4.29±0.25	4.97±0.35	3.55±0.32	5.38±0.22*	5.66±0.21*	5.47±0.30*
Total protein, g/L	73.3±1.1	72.9±1.2	76.5±1.6	74.1±2.1	72.3±1.0	74.6±1.1	74.7±0.5	74.8±2.1
Albumine, g/L	47.4 ± 0.7	45.4 ± 1.1	45.9± 0.5	45.0±0.6	44.1±0.7	42.8±0.8*	44.3±0.6	43.6±0.7*
Globuline, g/L	25.9± 1.0	27.5±1.1	30.6±1.8	29.0±2.1	28.2±1.0	31.9±1.5	30.4±0.1	31.2±1.7
Ratio albumin /globulin	1.88±0.08	1.69±0.08	1.56±0.08	1.65±0.11	1.59±0.07	1.39±0.09*	1.49±0.09	1.43±0.05*
Cholesterol, mM	2.38±0.11	2.38±0.11	2.65±0.07	2.54±0.08	2.25±0.09	2.69±0.13	2.87±0.11*	2.83±0.10*
Triglycerides, mM	0.34±0.03	0.32±0.03	0.42±0.05	0.54±0.12	0.30±0.03	0.39±0.02	0.42±0.03	0.40±0.05
Non-esterified fatty acids, mM	0.28±0.08	0.40±0.12	0.10±0.01	0.09±0	0.09±0	0.12±0.02	0.09±0	0.10±0.01
Sodium, mM	143±2	142±2	143±2	144±1	143±2	144±2	142±2	142±2
Potassium, mM	4.75±0.07	4.64±0.09	4.84±0.12	4.76±0.09	4.85±0.09	4.84±0.08	5.07±0.12	4.69±0.11
Chloride, mM	95.5±0.4	96.0±0.6	96.7±0.6	96.7±0.6	97.0±0.4	96.6±0.4	96.6±0.5	97.1±0.4
Calcium, mM	1.16±0.02	1.15±0.02	1.20±0.01	1.19±0.02	1.18±0.01	1.23±0.01	1.17±0.02	1.17±0.01
Anorganic Phosphorus, mM	2.70±0.04	2.67±0.05	2.60±0.03	2.59±0.05	2.68±0.04	2.63±0.04	2.62±0.04	2.48±0.04*

Values are given as mean ± SE; n=15, except for CoVaccine-0, -400 and -800 (n=14); * Means differ from that of the corresponding PBS control group ($p<0.05$).

Table 5: Histological evaluation of injection sites per treatment of pigs sacrificed at week 12[a].

Measure	PBS controls	ISA-0	ISA-400	ISA-800	CoVaccine-0	CoVaccine-400	CoVaccine-800
No findings	12	9	1		2	4	4
Mild lymphocytic inflammation	3	2			1	2	
Mild granulomatous inflammation			1	1	3	5	9
Moderate/severe gran.ulomatous inflamm.		4	6	7	4	2	1
Fibrosis			2	3	5	2	1
Suppurative inflammation			5	4			

[a] n=15.

After the first immunization, no clinical symptoms were seen for any of the treatments. However, transient edema was observed in all animals treated with CoVaccine formulations (CoVaccine-0, -400 and -800) after the second immunization and in 34 out of 45 animals after the third immunization. This moderate edema occurred at day 2 to 4 after immunization and lasted for at least 4 days, while it had disappeared or started to disappear by the end of the observation period, i.e. 10 days after the successive immunizations. An increase in body temperature of approximately 1°C ($p<0.05$) was observed in the CoVaccine-400 group at day 2 and 4 after the second and third immunization and at day 4 after the second and third immunization in the CoVaccine-0 and CoVaccine-800 groups. At day 6 after immunization body temperatures had returned to normal (data not shown).
In the ISA-400 and ISA-800 group, red coloring and tissue swelling occurred at day 2-4 after the second and third immunization in about 70% of the animals and lasted for 6-8 days in about 50% of the affected animals and persisted for more than 10 days in the remaining animals. Only a few animals in the ISA-0 group showed minor local reactions. No changes in body temperature were detected in the ISA-treated animals.
Following autopsy in week 12, 20 and 30, the 3 injection sites of each pig were examined histologically. The observations of the injection sites of the animals sacrificed at week 12 are summarized in Table 5. In the PBS group 3 out of 15 injection sites displayed minor reactions. In the ISA-0 group, 2 sites showed minor lesions and 4 sites moderate or severe lesions, all localized in the musculus semitendinosus. In case of the animals treated with ISA-400 and ISA-800, 27 out of 30 injection sites showed moderate or severe granulomatous inflammation, fibrosis or suppurative inflammation. In the CoVaccine treated animals, 10 injection sites had no detectable lesions, while 20 sites with mild and in 15 sites with moderate inflammatory reactions were detected. Similar results were noted for injection sites of pigs sacrificed at week 20 and 30, indicating that these lesions are persistent. The following exceptions were observed: at week 20, severity scores of injection sites of ISA-0 pigs were in between the severity score of ISA-400, ISA-800 and CoVaccine-0,-400 and -800. At week 30, only a few abnormalities were noted in PBS controls and CoVaccine-800 treated animals. The severity of the side effects could be rank-ordered as follows: ISA-800 > ISA-400 > ISA-0 > CoVaccine-0 > CoVaccine-400 > CoVaccine-800 > PBS controls (data not shown).

4. DISCUSSION

In this study potential GnRH vaccines for possible human application were evaluated for chronic toxicity and efficacy. The vaccines contained 400 or 800 μg antigen in ISA 51 adjuvant or in CoVaccine adjuvant. Several clinical trials have confirmed that ISA 51 is a safe and tolerable adjuvant for human vaccines (Gringeri et al., 1998; Pinto et al., 1999;

Van Driel et al., 1999; Slingluff et al., 2001). However, vaccines comprising ISA 51 have not yet been approved for clinical use. CoVaccine is a newly developed adjuvant for human therapeutic vaccines with an high activity against various antigens (Blom and Hilgers, 2004). Application of the latter adjuvant in a GnRH immunization study in horses resulted in high antibody responses and a rapid decrease in serum testosterone concentrations to undetectable levels (Turkstra et al., 2005).
With both doses of antigen, CoVaccine appeared to be more effective than ISA 51. Testosterone depletion was observed at 4 weeks after the initial immunization in 50% of the animals treated with CoVaccine-400 and CoVaccine-800, whereas at week 8 testosterone levels were below the detection limit of the assay in all animals of these groups. Testosterone levels continued to remain undetectable until the animals were sacrificed at weeks 12, 20 or 30. In contrast, in ISA-400 and ISA-800 treated animals, testosterone concentrations were at castrate levels in only 13 out of 30 animals at week 8, whereas only one of these animals showed an early response with undetectable testosterone levels at week 4 of the study. A 'late' testosterone depletion at week 13 or later was noted in 5 pigs. LH concentration in plasma were not significantly affected, except in the CoVaccine-800 group at week 12 and 20. However, mean LH levels seemed to be lower in all GnRH immunized groups from week 4 onwards. Moreover, LH concentrations in animals with undetectable testosterone levels were always less than 1.7 ng/ml, while in the controls and non-responding animals, LH levels ranged from 1.0 to 3.9 ng/ml. FSH levels appeared to be lower in CoVaccine-400 and CoVaccine-800 treated animals than in the control groups from week 8 till the end of the study, suggesting an small inhibiting effect of GnRH immunization on plasma FSH concentration. However, these effects were not confirmed statistically. Effects of GnRH immunization on FSH levels in pigs differ among studies. In some studies in young male pigs no effects were observed (Awoniyi et al., 1988; Wagner and Claus, 2004), whereas others found a small but significant reduction in male (Caraty and Bonneau, 1986) and female pigs (Esbenshade and Britt, 1985). The variable effects of GnRH immunization on FSH levels could be due to the fact that FSH may respond to a releasing factor other than GnRH. Recently, a GnRH isoform with an amino acid sequence similar to lamprey GnRH-III was found to selectively activate FSH release in rats and bovines (Yu et al., 1997; Dees et al., 2001; McCann et al., 2001), although this finding was not corroborated in bovines by others (Amstalden et al., 2004). Depending on the GnRH antigen used for immunization, the induced antibodies may or may not neutralize the GnRH isoform responsible for FSH activation. Indeed, antibodies raised against the GnRH antigen used in this study showed cross-reactivity with lamprey GnRH-III and thus may have an additional inhibitory effect on FSH levels (Turkstra et al., in press).
The mean sperm number and sperm quality was significantly lower in all GnRH immunized groups compared to PBS controls. These effects were more pronounced in CoVaccine-400

and CoVaccine-800 treated animals than in the ISA-400 and ISA-800 groups. Sperm number and sperm quality correlated with testis weight and plasma testosterone concentrations, which were also lower in the CoVaccine-400 and CoVaccine-800 groups compared to the ISA-400 and ISA-800 groups.
Sperm quality was affected only in treated animals with sperm counts below 5 $x10^9$. This could be caused by the low testosterone levels in these animals, assuming that below a certain threshold value spermatogenesis is not only affected quantitatively, but also qualitatively. Another explanation could be that in animals in which sperm production is drastically reduced, the sperm flow from the testis to the cauda is strongly diminished, resulting in aged sperm in the cauda. Indeed, in animals with a very low sperm output and more than 50% dead sperm, we observed a very high proporton of loose heads, suggesting aging of the cells rather than faulty spermiogenesis.
Although transient local tissue reactions were observed after the second and third immunization in animals treated with CoVaccine formulations, histopathological evaluation of the injection sites showed only minor morphological changes in the muscle tissue. In contrast, moderate and severe inflammatory reactions were detected in animals treated with ISA-400 and ISA-800.
Edematous reactions with CoVaccine have not been seen in previous studies in which the vaccine was injected in the neck region (unpublished results). This suggests that this reaction is typical for injection in the musculus semitendinosus. An explanation could be that, after injection of the vaccine in the neck region, the vaccine depletes intermuscularly, whereas in case of injection in the musculus semitendinosus the vaccine is enclosed in the muscle tissue, causing this reaction. Moreover, compared to the neck muscles, the musculus semitendinosus is almost constantly activated, which may enhance the reaction.
Immunization with the G6k-GnRH-tandem-dimer-KLH conjugate in CoVaccine or ISA 51 adjuvant did not induce changes in weight of non-reproductive organs, while also no macroscopic abnormalities were observed in these tissues. The presence of GnRH mRNA and the GnRH receptor mRNA in non-reproductive tissues may suggest that GnRH actions are not limited to the hypothalamus-pituitary-gonadal axis. However, in accordance with the data of the present study, no dramatic effects of GnRH immunization on non-reproductive organs or organ functions have been reported by others (Gual et al., 1997; Kumar et al., 2000). Only minor effects on organ weights have been observed in rats. GnRH immunization decreased adrenal and pituitary weight, but did not induce morphological changes in these organs (Kumar et al., 2000). The reduced adrenal weight may be due to the sensitivity of rats for GnRH immunization (Ferro et al., 2004), resulting in complete depletion of GnRH and LH from circulation. As receptors for GnRH and LH have been found in rat adrenals (Kakar et al., 1994; Apaja et al., 2005), the absence of these hormones may affect adrenal development or function.

As GnRH immunization in the rat decreases circulating gonadotropins to undetectable levels (Awoniyi et al., 1992; McLachlan et al., 1994), it is likely that the LH and FSH content in the pituitary is reduced too, as has been demonstrated in other species (Adams and Adams, 1986; Rabb et al., 1990). This may in part explain the reduction of pituitary weight in rats. Although, GnRH immunization reduced gonadotropin concentration and pituitary weight in rats, other pituitary hormones were not affected (Awoniyi et al., 1993; Rovan et al., 1992).
Reduced weights of heart, liver and kidneys as observed in GnRH immunized rats, are presumably due to androgen deprivation, as similar effects were found in surgically castrated rats (Ferro et al., 1996; Kumar et al., 2000). In pigs (this study) and in rabbits (Kumar et al., 2000) none of these effects were noted.
Evaluation of hematological, biochemical and urine parameters revealed no striking effects of GnRH immunization. However, androgen depletion increased urea concentrations in plasma of surgically castrated pigs and pigs of the CoVaccine-400 and CoVaccine-800 groups. Similar effects were observed in surgically and hormonally castrated male deer, bovines and humans (Morris and Bubenik, 1983; Doornenbal et al., 1987; Nishiyama et al., 2005). This can be explained by the fact that anabolic effects are diminished due to testosterone deprivation by castration or GnRH immunization. As a consequence, feed intake is higher than the amount required for maintenance of metabolic functions and growth, resulting in an excess of amino acids that need to be catabolized. This results in an increase in blood urea concentration and nitrogen excretion (Coma et al., 1995, Metz et al., 2002).

5. CONCLUSION

In this study we observed that immunization with the G6k-GnRH-tandem-dimer-KLH conjugate in CoVaccine resulted in a rapid decline of plasma testosterone levels, while effects of the GnRH-KLH conjugate in ISA 51 on testosterone levels were highly variable. No acute toxic effects were observed, although transient injections site reactions were observed after CoVaccine injection in the musculus semitendinosus. Chronic toxicology evaluation did not reveal remarkable alterations. Thus, immunization of male pigs with the G6k-GnRH-tandem-dimer-KLH conjugate in CoVaccine adjuvant is a highly effective method to reduce plasma testosterone concentration to undetectable levels and does not induce chronic toxic effects.

6. ACKNOWLEDGEMENTS

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Chapter 9

General discussion

Immunization against GnRH as a method to reduce gonadal steroid secretion has been studied for many purposes. In farm animals it has been used to improve growth performance, meat quality and handling of the animals and to prevent pregnancies and undesired sexual behavior (Ladd et al., 1994; Bonneau and Enright, 1995; Jago et al., 1997; Millar et al., 1997; Millar et al., 2000; Dunshea et al., 2001; Zeng et al., 2002b; Zeng et al., 2002c; Price et al. 2003; Robbins et al., 2004). In humans, immunization against GnRH has potential for treatment of endocrine disorders (Gaul et al., 1997) and treatment of gonadal steroid-dependent tumor growth (Ferro and Stimson, 1997; Talwar, 1997; Jacobs et al., 1999; Simms et al., 2000).
So far, implementation of GnRH vaccines in practice has been hampered mainly because of the low efficacy of the vaccines, i.e. the inability to raise a sufficient immune response in order to block gonadal steroid production in each vaccinated individual. Various attempts have been made to solve these problems, like modification of the antigen, the use of more effective carrier molecules and more immunostimulatory adjuvants. Since the choice of adjuvants and carrier molecules is rather limited, we have started the present study. Methods have been developed to enhance the immunogenicity of the antigen itself. It was shown that the immunogenicity of the native GnRH molecule can be increased by enlargement of the GnRH peptide to a tandem molecule, whereas it becomes even more immunogenic when the tandem peptide is dimerized and non-native amino acids are introduced. Moreover, the specificity of the antibodies can be further modified by amino acid replacements within the antigen itself. The optimized antigen appeared to be fully effective in combination with different carrier proteins and adjuvants in pigs. GnRH vaccination in pigs improved growth performance as compared to surgical castrated pigs. In horses, full efficacy was obtained with the optimized antigen in a well tolerated adjuvant. The vaccine is not toxic. A chronic toxicity study performed in pigs, in order to be able to start a Phase 1 clinical trial in prostate cancer patients, showed that no significant abnormalities were induced after short-term and long-term exposure to high levels of GnRH neutralizing antibodies.

Increased immunogenicity of GnRH-tandem peptides

A highly efficient GnRH vaccine was developed by enlarging the GnRH molecule to a GnRH-tandem construct (Meloen et al., 1994). In chapter 3 we showed that the GnRH-tandem and GnRH-tandem-dimer peptides are immunogenic by itself and immunosterilized, i.e. block testes growth and function in male piglets effectively (immunosterilization was previously called immunocastration, however as the testes are not removed but only temporary inactivated, immunosterilization is a more appropriate name). Immunogenicity was further increased by conjugation of the peptides to a carrier protein. Conjugation of a GnRH-tandem-dimer peptide, in which a glycine at position 6 was

substituted by a D-lysine, to ovalbumin (OVA) resulted in a highly effective antigen (G6k-GnRH-tandem-dimer) which appeared to be slightly more effective than the originally prepared GnRH-tandem peptide (Figure 1). The G6k-GnRH-tandem dimer peptide conjugated to OVA and emulsified in Specol adjuvant showed the highest efficacy with 9 out of 9 pigs responding, as determined by very low testes weights. A dose response study with this vaccine revealed that even with peptide doses as low as 5 µg almost complete effectivity could be obtained (chapter 3). This vaccine has been tested extensively in male piglets. Vaccination with peptide doses ranging between 50-150 µg emulsified in Specol adjuvant substantially inhibited testis growth in 98% out of a total of 200 vaccinated pigs (unpublished results). In Chinese cross-bred pigs, the vaccine also caused a reduction in testis weight in 35 out of 39 treated male pigs (Zeng et al., 2001; 2002a), whereas 10 out of 12 vaccinated female pigs showed decreased LH and inhibine levels and a reduction in ovarian weights (Zeng et al., 2002b).

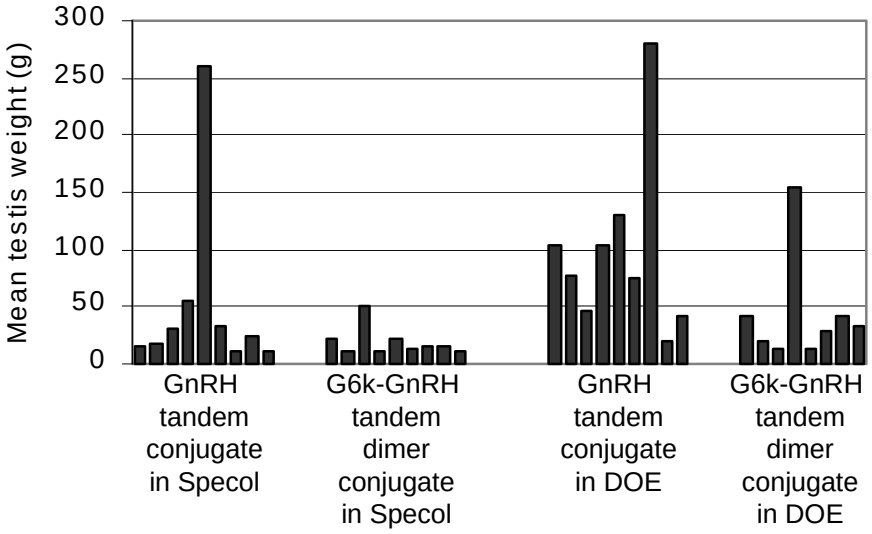

Figure 1: Mean testes weight of pigs following immunization with GnRH-tandem-OVA conjugate or G6k-GnRH-tandem-dimer-OVA conjugate in Specol adjuvant or Double Oil Emulsion (DOE). Testis weight of untreated controls ranged between 200 and 300 g. Responders are defined as pigs with a testis weight less than 150 g (Oonk et al., 1995)

Molecular alterations: effects on efficacy and specificity

In chapter 4 the role of the individual amino acids of the G6k-GnRH-tandem-dimer was investigated. This was done by replacing each amino acid of the decapeptide one at the time by alanine. These constructs were evaluated for their ability to establish immunosterilization in male piglets. It was shown that 4 out of 9 amino acids (amino acids at position pE1, S4, R8 and G10 of the decapeptide of the G6k-GnRH-tandem-dimer) can be individually replaced by alanine without affecting the efficacy of immunosterilization. Replacement of amino acids in 3 other positions (position Y5, L7 and P9) resulted in a reduction of the efficacy, as 5, 6 and 6 out of 7 piglets were immunosterilized, respectively. Replacements at position H2 and W3 did not lead to immunosterilization. These results indicate that several amino acids can be replaced in order to make the peptide more ´foreign´, without affecting the ability to induce the formation of GnRH neutralizing antibodies.

In the past few years, the presence of several non-mammalian GnRH-isoforms have been demonstrated in mammals (White et al., 1998; Dees et al., 1999; Urbanski et al., 1999; Yahaloma et al., 1999). These GnRH-isoforms (GnRH-II, salmon GnRH and 2 forms of lamprey GnRH) most likely play a limited role in the regulation of fertility in mammals (Yahaloma et al., 1999; Cheon et al., 2001; Siler-Khodr and Grayson, 2001). However, GnRH-II may have functions which are not related to reproduction, as suggested by the presence of high concentrations of GnRH-II in the kidneys (White et al., 1998), an increase in kidney weight in rats immunized against GnRH-II (Ferro et al., 2001) and the widespread expression of GnRH-II receptor mRNA (Neil, 2002). Immunization against GnRH, in order to block gonadal steroid secretion, may induce the formation of antibodies that cross-react with these GnRH-isoforms, which may result in undesired side effects due to neutralization of these isoforms. In chapter 5 we show that the cross-reactions can be circumvented by using appropriate engineered antigens. Substitution of pyroglutamine at position 1 or serine at position 4 of the G6k-GnRH-tandem-dimer peptide by alanine resulted in an immunogenic antigen, which induced the formation of antibodies that neutralised only mammalian GnRH (GnRH-I) and established immunosterilization in male piglets, without showing cross-reactivity with other GnRH-isoforms (Turkstra et al., 2001). In general, isoform specific antibodies can be generated by using a peptide containing the isoform specific region, i.e. the C-terminal part of the GnRH decapepide, while the N-terminal part of the decapeptide, is altered by introducing a non-native amino acid. This approach has also been applied successfully by Ferro et al. (2001), who substituted the N-terminal pyroglutamine by a cysteine, resulting in the formation of GnRH antibodies, which did not cross-react with GnRH-II.

GnRH immunization in pigs

Efficacy and side effects of Specol and alternative adjuvants

The choice of the adjuvant is limited by the side effects induced. Strong adjuvants, in particular water-in-mineral oil emulsions, may cause strong and persistent inflammatory reactions. In both human and veterinary field, the risks of adjuvanted vaccines must be compensated fully by the benefits of the treatment (EMEA ´note for guidance´ CVMP/IWP/043/97). Although high immune responses were generated with the G6k-GnRH-tandem-dimer in Specol adjuvant, local reactions at the site of injection were noted. In a comparative study in young male piglets, various types of adjuvants were evaluated for efficacy and side effects (unpublished data). The following adjuvants were studied: Specol (water-in-mineral oil emulsion; ID-DLO, Lelystad, The Netherlands), Suvaxyn O/W (mineral oil-in-water emulsion; Fort Dodge Animal Health, Weesp, The Netherlands), SL-CD/Squalane, which is an aqueous formulation comprising sulfolipocyclodextrin and squalane (kindly provided by Fort Dodge Animal Health, Weesp, The Netherlands), and Carbopol 934P, comprising polyacrylic acid polymers (BFGoodrich, USA). Antigen in phosphate buffered saline (PBS) served as a negative reference.

Immunization with Specol or SL-CD/Squalane resulted in reduced testis weights and high antibody titers in all animals (Table 1). Suvaxyn was less effective, though testis growth was inhibited. The antigen administered in Carbopol and PBS, reduced testis weights in some animals.

Table 1. Efficacy of G6k-GnRH-tandem-dimer OVA conjugate in different adjuvants

Adjuvant	No. of responding pigs per total no.	Testis weight responding pigs, median (range)	Testis weight non-responders, median (range)	Antibody titer in GnRH ELISA[$], (mean ± SD).
Specol	10/10	25 (11-95)	-	3.0 ± 0.2
Suvaxyn	9/10	60 (22-159)	324*	1.9 ± 0.3
SL-CD/Squalane	10/10	31 (15-73)	-	3.1 ± 0.3
Carbopol	2/10	63 (30-96)	282 (248-363)	1.4 ± 0.2
PBS	1/10	67	282 (243-355)	1.3 ± 0.5

[$]Antibody titre of sera obtained 3 weeks after the booster vaccination was calculated as the log of the dilution factor which gave an optical density of 1500

* Weight of the descended testis of a cryptorchid boar.

Examination of the injection sites after autopsy revealed that the majority of the injections sites (17 out of 20) of Specol treated pigs showed lesions, consisting of fibrous tissue formation, granulomatous inflammation and in some cases a purulent inflammation or abscess formation. In the Suvaxyn as well as in the SL-CD/Squalane treated pigs, 60% of the injection sites could

not be localized due to the absence of any tissue lesions. The lesions noted were much smaller than in the Specol treated pigs and consisted of connective tissues located intermuscularly. In the remaining groups no tissue reactions were observed. These data indicate that G6k-GnRH-tandem-dimer OVA conjugate in Specol is an effective vaccine but elicits adverse reactions. These side effects can be circumvented by using Suvaxyn or SL-CD/Squalane as adjuvant, while the desired biological effects are largely maintained.
In conclusion, G6k-GnRH-tandem-dimer OVA conjugate is highly immunogenic and allows the use of a broad range of adjuvants to effectively immunosterilize male piglets.

Alternative for surgical castration
Worldwide, every year hundreds of millions male pigs are surgically castrated. Castration is performed to prevent the occurrence of boar taint, an unpleasant odour which becomes noticeable when the meat is heated. During the last decade, politicians, veterinarians, scientist and animal welfare organizations in Europe have argued against this animal unfriendly method of surgical castration. It is painful for the animals and causes stress (Wemelsfelder and Van Putten, 1985, McGlone and Hellman, 1988, McGlone et al., 1993, White et al., JAS 1995). Moreover, surgical castration affects the health of the pigs, it suppresses the immune system (Lessard et al., 2002) and accounts for a doubling of the incidence of pneumonia, pleuritis and pericarditis at slaughter (De Kruijf and Welling, 1988, Van der Peet-Schwering and Swinkels, 1987). Alternatives for surgical castration as described in chapter 2, all have major disadvantages and are not likely to be implemented in the near future. Vaccination against GnRH provides an alternative, which circumvents the animal unfriendly aspects of surgical castration. In addition, it improves growth performance (Bonneau et al., 1994; Dunshea et al., 2001; Zeng et al., 2002c) making this approach even more profitable for the farmer.

GnRH immunization improves growth performance in pigs
Several studies have demonstrated improved growth performance of pigs immunized against GnRH as compared to surgically castrated pigs (Bonneau et al., 1994; Dunshea et al., 2001; Zeng et al., 2002c). Due to the presence of androgens during a substantial part of the fattening period, growth performance characteristics of boars vaccinated against GnRH are similar to intact boars until androgen levels become severely reduced and effective immunosterilization apparent. In chapter 6 we showed that immunosterilized boars had a higher growth rate and grew more efficiently than surgically castrated pigs, while carcass quality, i.e. backfat thickness and meat percentage, was similar to intact boars. In addition, immunosterilized boars could easily be distinguished from intact boars at the time of slaughter by the size of the testes and the appearance of the scrotum. Testis size was reduced by 25% and the immunosterilized pigs exhibited a flat scrotal sac, while the

scrotum of intact boars had a bulbous appearance. Moreover, testis weight of the immunosterilized boars was reduced by 75% as compared to intact boars.
In our study, feed intake was restricted and similar for all animals. In case pigs have ad libitum excess to the feed, effectively immunosterilized pigs show a similar eating pattern as surgical castrates (Zeng et al. 2002c). Due to this high feed intake, more fat tissue is gained, resulting in lower carcass quality (Zeng et al., 2002c). For this reason surgically castrated pigs are generally fed restrictedly in the last weeks of the fattening period. This feeding regimen should also be applied to boars, immunosterilized relatively early during the fattening period to prevent the development of carcasses with a high fat thickness.
Feed efficiency (i.e. kg weight gain/kg feed) in boars effectively immunosterilized late in the fattening period (6 weeks before slaughter) was higher than in early immunosterilized boars and surgical castrates (chapter 6). Bonneau et al. (1994) reported that boars which received the second immunization 2 weeks before slaughter, showed a feed efficiency similar to intact boars. However, immune responses in some immunized pigs were rather low, indicating inefficient immunosterilization. Furthermore, when the second vaccination is given within 4 weeks before slaughter, testis weight can not be used as an indication for effective immunosterilization, as it overlaps with the testis weights in intact boars (Bonneau et al.,1994; Dunshea et al., 2001).
Although the most important reason to ban surgical castration in piglets is the improvement of animal welfare, the better growth performance of immunosterilized boars as compared to surgical castrates could also be an important additional aspect for the implementation of GnRH immunosterilization in pig husbandry.

Concerns about practical application of GnRH vaccination in piglets
Technically, vaccination against GnRH provides an attractive alternative for surgical castration in pigs: it effectively reduces the levels of both boar taint and it does not affect the well-being of the animal, as surgery is abolished. However, in the past decade in the Netherlands some reports/papers have been published in which the authors exhibited their concerns about the possible implementation of immunosterilization through vaccination against GnRH in pig husbandry. The main issues of these papers are summarized below.
Van Casteren (1995) considered vaccination against GnRH as less painful for the piglet as compared to surgical castration. However, he did not differentiate between both techniques with respect to the integrity of the piglet; according to the author vaccination against GnRH is in conflict with the integrity of the animal in a similar way as surgical castration, due to the fact that the animal loses its reproductive capacities.
Rutgers (2001) mentioned that immunisterilization interferes with the biological system of the pig and thus affects the ´pig-being´ (in dutch: ´het varken-zijn´) of the pig. According to the author, approval of immunosterilization would be a step further towards the pig being ´a

thing´ instead of an animal (in dutch: ´de verdinglijking van het dier´). Furthermore, this author mentioned that immunosterilization most likely will not be accepted by the consumer, as it will be associated with the application of hormones in pig husbandry. The Dutch Veterinary Society based their statement regarding surgical castration in pigs on the same aspects, integrity of the animal and consumer acceptance. The Society concluded that surgical castration as well as vaccination against GnRH should be rejected, while more effort should be put into other alternatives (Anonymous, 1999).
Others, who considered GnRH vaccination as a suitable alternative for surgical castration without anaesthesia, argued against this attitude (Meloen, et al., 1999; Strikwerda, 1999; Wijsmuller, 2001; Strikwerda, 2001; Wensing and Turkstra, 2001). Moreover, the Federation of Veterinarians of Europe concluded that vaccination against GnRH could be a socially viable alternative for surgical castration, depending on the acceptance given by the consumer (FVE position paper, FVE/01/083, 2001).
With respect to the ethical and social aspects of a possible implementation of vaccination against GnRH as mentioned above, the following remarks can be made: vaccination against GnRH is an animal friendly technique, in particular when compared to the current castration practice. Moreover, it causes only a temporary suppression of hormone levels of LH, FSH and gonadal steroids, while the pig maintains intact. Implementation of GnRH vaccination in pig husbandry, therefore, can be seen as a step forward, leading to a more animal friendly pig husbandry. Consumers should be informed about the current practices in pig husbandry and its consequences. Surgical castration, for instance, results in extreme high levels of LH and FSH, while immunosterilization reduces LH and FSH levels. In this context, meat of immunosterilized pigs can be considered as ‘hormone-poor’ meat. Moreover, surgical castration affects the health of the pigs (de Kruijf and Welling, 1988; Lessard et al., 2002). In conclusion, objections against GnRH vaccination in pigs are not to be expected or will be at least toned down, provided that the consumer is objectively informed about this way of preventing boar taint in pigs.

Future outlook
It is expected that the EU will approve a GnRH vaccine for prevention of boar taint in the near future. It is a suitable and animal friendly alternative for surgical castration, which effectively reduces boar taint and improves growth performance.

GnRH immunization in horses
In horses, vaccination against GnRH could be a solution for suppression of sexual behavior in both stallions and mares. Other treatments, such as GnRH agonist or antagonist or steroid application have been shown to be inadequate, costly or prohibited at certain occasions

(Stout and Colenbrander, 2004), while castration carries surgical risks. However, application of GnRH vaccination in horses, is hampered by inefficiency of the vaccines (Malmgren et al. 2001, Dalin et al., 2002), as well as the occurrence of side effects due to vaccination (Dowsett et al., 1991; Malmgren et al., 2001). Recently, a GnRH vaccine (Equity ™, CSL Animal Health) became available in Australia to control estrus and estrus-related behavior in mares. According to the medical guide, the vaccine is not effective in all mares, emphasizing the difficulty to develop an effective vaccine for the use in horses.
The choice of the adjuvant is of major importance for the efficacy of the vaccine. However, it also determines the severity of the side effects. In horses, mineral oil adjuvants are effective, but these have been shown to induce severe injection site lesions (Dowsett et al., 1991). In chapter 7 we showed that CoVaccine™ HT adjuvant is well tolerated and effective in horses. The vaccine reduced serum testosterone levels in all 4 treated stallions from week 6 until the end of the study (week 14). Testis function was affected as determined by reduced seminiferous tubule size, affected spermatogenesis, atrophy of the Leydig cells and a reduction in sperm quality. In another second study (Van der Meer et al., 2001), the CoVaccine™ HT vaccine reduced testosterone levels, sperm production and sperm quality for 4-5 months in 3 out of 5 treated stallions. The remaining 2 stallions responded moderate, testosterone levels in these animals were reduced for 6 weeks. The study further showed that the effects of immunization were reversible within 8 months after the second vaccination.
Despite the distinct effects on testosterone levels and testes function in young sexually mature stallions, effects may be limited in older stallions (Stout and Colenbrander, 2004). In conclusion, the use of the G6k-GnRH-tandem-dimer OVA CoVaccine™ HT vaccine in sexually mature stallions is well tolerated and causes a reduction in testosterone levels, subsequently leading to affected testis function and sperm quality. However, the variation in duration of the response is a point of attention and needs to be further studied.

GnRH immunization in humans

Chronic toxicity and efficacy study in pigs

In order to initiate a clinical trial in humans, a chronic toxicity study was performed in pigs. Two candidate vaccines, containing the G6k-GnRH-tandem-dimer peptide conjugated to the carrier protein Keyhole Limpet Hemocyanin (KLH) in ISA51 adjuvant or in CoVaccine™ HT adjuvant were evaluated. Immunization with the GnRH-CoVaccine formulations led to undetectable testosterone levels 8 weeks after the initial immunization in all 30 animals treated. Responses in GnRH-ISA 51 treated animals were less consistent: 13 out of 30 animals showed undetectable testosterone levels at week 8 after the initial vaccination, while in 5 animals testosterone decreased at a later stage. Testosterone

deficiency led to atrophy of the testes and accessory sex organs at 12, 20 and 30 weeks after the initial vaccination. After the second and third vaccination, transient local tissue reactions were seen at the site of injection in all animals treated with CoVaccine formulations. However, at autopsy, only minor injection site lesions were noted in some but not all animals, while lesions were moderate in GnRH-ISA 51 treated animals. Androgen depletion increased urea concentrations in plasma of surgical castrates and pigs of the CoVaccine-400 and CoVaccine-800 groups, presumably due to a higher feed intake than required for maintenance and growth (Coma et al., 1995; Metz et al., 2002). No effects on organ weights were observed in immunized animals and no systemic toxicological effects occurred. In conclusion, the G6k-GnRH-tandem-dimer KLH conjugate can cause a rapid reduction in serum testosterone, in particular in combination with CoVaccine adjuvant. Both vaccines were not toxic; no significant abnormalities were observed in the pigs after short-term and long-term exposure to high levels of GnRH neutralizing antibodies.

Clinical trials

As mentioned in Chapter 2, several companies have developed GnRH vaccines for treatment of gonadal steroid-dependent cancers, mainly prostate cancer, and tested these vaccines in clinical trials. However, none of the performed clinical trials have been completed successfully, despite encouraging results in animal studies. The main reason for this drawback, is a lack of full efficacy. This may be caused by the use of weak adjuvants, low antigen doses or low immunogenicity of the antigen. In addition, poor responses in men could also be caused by a compromised immunity of the patient due to age and prolonged medical treatment before GnRH vaccination was offered as a treatment (Agatsuma et al., 1979; Dunn-Walters et al., 2003). We believe that the insufficient immunogenicity of the antigens described is the major problem, as suggested by the increased immunogenicity obtained with GnRH-tandem and GnRH-tandem-dimer conjugates as compared with GnRH-monomer conjugates.

Future outlook

GnRH vaccination in prostate cancer patients could be a therapy with advantages over the currently used therapy. It does not require the use of additional medication to prevent side effects, like GnRH agonist treatment (see Chapter 2), and costs of treatment are low. GnRH antagonist therapy could also be a promising means to reduce androgen levels in prostate cancer patients, however high peptide doses and frequent administration are expected to be required to maintain this status. Moreover, undesired side effects may occur. Therefore, GnRH vaccination seems to be a more cost-effective and patient friendly approach for future treatment of these patients.

Overall conclusion

Vaccination against small endogenous peptides suffers from a lack of immunogenicity which can be compensated at least partially by the use of strong adjuvants. As only a limited number of adjuvants are allowed for application in practice, modification of the antigen in order to improve the immunogenicity of the vaccine is of major importance to develop an effective vaccine. We improved the immunogenicity of the small endogenous peptide GnRH by enlarging the molecule to a tandem peptide comprising twice the amino acid sequence of the native peptide. Dimerization of this peptide and introduction of foreign amino acids further enhanced its immunogenicity. Final optimization was established by conjugation of the optimised peptide to a efficient carrier protein. A variety of vaccine formulations, all containing the optimised GnRH-tandem-dimer, were effective and well tolerated in several mammalian species.

Perspectives

Improving immunogenicity by 'multimerization' and amino acid substitutions has been successfully applied for GnRH and will eventually lead to a marketable vaccine. This approach can be extrapolated to many other peptides and proteins, which are of interest due to their role in tumor development and cancer. These so-called soluble mediators display a trophic effect on tumor growth by endocrine, paracrine or autocrine actions. Neutralization of these keyplayers by active immunization using highly immunogenic modified antigens, may offer an appropriate and cost-effective way to block tumor growth. Currently, therapeutic vaccines against soluble mediators in pancreatic cancer and breast cancer are being developed.

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Summary

Gonadotropin-releasing hormone (GnRH) plays a pivotal role in fertility and reproduction in mammals. It induces the release of luteinising hormone (LH) en follicle stimulating hormone (FSH) from the pituitary. These hormones are responsible for gonadal steroid production and indirectly for gametogenesis.
GnRH is a small peptide hormone, which can be produced syntheticly; it has been applied for treating fertility-related disorders and gonadal steroid-dependent cancers in human and veterinary medicine. Due to its short half-life, large amounts of GnRH peptide have to be administered, which makes it a costly treatment. In addition, these GnRH analogs may cause side effects and are not effective in all species.
A vaccine against GnRH could offer a cheap, long term effective and safe alternative. However, development of GnRH vaccines is hampered by the lack of efficacy. Even the common approach to increase immunogenicity, i.e. by coupling of the peptide to a carrier protein and the use of strong adjuvants, is not sufficient to induce a GnRH neutralizing immune response in all vaccinated individuals. Since the choice of adjuvants and carrier molecules is rather limited, we have initiated this study in order to develop a vaccine in which the immunogenicity of the antigen itself is increased.
In this thesis, studies are presented which described the development of a highly immunogenic GnRH antigen, as well as the evaluation of this antigen in vaccines in target species.
In chapter 2 an overview of the literature on GnRH immunization is presented.
Chapter 3 describes the development of a highly immunogenic GnRH antigen. The GnRH peptide was enlarged to a GnRH-tandem peptide and immunogenicity was further increased by dimerization of the peptide and introduction of foreign amino acids (G6k-GnRH-tandem-dimer). This modified peptide construct was conjugated to ovalbumine. It appeared to be highly effective in immunosterilization, i.e. blocking testes growth and function, in male piglets.
In chapter 4, the role of the individual amino acids of the G6k-GnRH-tandem-dimer peptide on immunosterilization efficacy was established. Therefore, each amino acid of the decapeptide was replaced one at the time by alanine. It appeared that amino acids at position 1, 4, 8 and 10 could be individually replaced by alanine without affecting efficacy of the vaccine, while replacement of amino acids at position 5, 7 and 9 only slightly

affected vaccine efficacy. In contrast, replacement of amino acids at position 2 and 3 did not result in immunosterilization in pigs, indicating that these amino acid are essential for inducing an GnRH neutralizing immune response. Furthermore, amino acids which can be replaced without affecting immunosterilization efficacy, can be replace in order to make the peptide more 'foreign' and thereby increasing immunogenicity.
Several GnRH-isoforms have been identified in mammals, however, the functions of these isoforms have not been elucidated yet. In order to avoid possible side effects due to neutralization of these isoforms by GnRH vaccination, we have tested antisera raised against a panel of G6k-GnRH-tandem-dimer antigens for their ability to bind the various GnRH-isoforms in vitro (chapter 5). Most of these GnRH antigens generated antibodies which bound the majority of the GnRH-isoforms, whereas two specific GnRH antigens raised antibodies which bound GnRH, but not the GnRH-isoforms. Thus, GnRH-specific antibodies can be raised using appropriate engineered antigens.
Immunosterilization by vaccination against GnRH could be a suitable alternative for surgical castration in male piglets. Worldwide hundreds of millions male piglets are surgically castrated during the first weeks of their lives to prevent the occurence of boar taint in the meat at time of slaughter. Boar taint is caused by androstenone and skatole, and associated with sexual maturity of the pig. Surgical castration prevents boar taint, but is an animal unfriendly practice, as it is performed without anaesthesia. It is painfull for the animal, causes stress and the animal becomes more susceptible for diseases. Immunisterilization is an animal friendly alternative for surgical castration and may also be beneficial for the growth performance. In Chapter 6, effects of the time of onset of effective immunosterilization on growth performance were studied. Growth performance of pigs, which were immunosterilized early or late during the fattening period were assessed. Effective immunosterilization early during the fattening period resulted in a growth performance similar to surgical castrates. In contrast, when immunosterilization was initiated late during the fattening period, growth performance was improved over surgical castrates and carcass quality appeared to be similar to intact male pigs. In addition, immunosterilized pigs of both groups had undetectable androstenone levels and could easily be distinguished from intact male pigs by the size of their testes.
In chapter 7, effects of GnRH vaccination were studied in sexually mature male ponies. Two vaccines with the G6k-GnRH-tandem-dimer antigen in CoVaccine and Carbopol adjuvant were evaluated. Both adjuvants were well tolerated. Antibody responses in the GnRH-Carbopol group were low or intermediate, resulting in a transient reduction in testosterone levels in only one of the four stallions treated. In contrast, all CoVaccine-treated stallions showed a high antibody response after the second vaccination, subsequently leading to undetectable testosterone levels and affected testis function.

In humane medicine, GnRH vaccination could be an alternative for hormone therapy in prostate cancer patients, as gonadal steroids act as a growth factor for the tumor. At present, hormone therapy includes GnRH agonists and anti-androgens, which block the production of gonadal steroid secretion. However, hormone therapy requires frequent administration, is costly and induces side effects. In order to be able to start a Phase 1 clinical trial with the GnRH vaccine in prostate cancer patients, a chronic toxicity study in pigs was performed. Two potential vaccines with the G6k-GnRH-tandem-dimer antigen in ISA 51 and in CoVaccine adjuvant were studied. The GnRH antigen in CoVaccine adjuvant appeared to be more effective than the antigen in ISA 51. All pigs treated with the former vaccine, showed undetectable testosterone concentrations, reduced weights of testes and accessory sex glands and substantially impaired sperm production and sperm quality. In the latter group, 18 out of 30 pigs responded. Injection with CoVaccine adjuvant led to a transient tissue reaction after the second and third immunization at the site of injection. However, only minor injection site lesions were seen at autopsy, while moderate injection site lesions were detected in the ISA 51 treated animals. Both vaccines can be considered as non-toxic; no chronic abnormalities were observed in blood, urine, organs or tissues.
In chapter 9, the results of the studies described in this thesis are summarized and possible applications are discussed.
The most important conclusion of the work presented in this thesis is that the G6k-GnRH-tandem-dimer peptide forms the basis of an effective GnRH vaccine with multiple applications. In the veterinary field, it could be a suitable alternative for surgical castration in pigs, eventually leading to a ban on this animal unfriendly practice in the EU. In human medicine, GnRH vaccination could replace hormone therapy in diseases driven by gonadal steroids. In prostate cancer for instance, GnRH vaccination may be a long term effective, cheap and safe alternative.

Samenvatting

Introductie
Gonadotropine-releasing hormoon (GnRH) speelt een belangrijke rol bij de regulatie van de voortplanting en het daarbij behorende gedrag bij zoogdieren. Het is verantwoordelijk voor de productie en afgifte van luteïniserend hormoon (LH) en follikel stimulerend hormoon (FSH) door de hypofyse. LH en FSH op hun beurt zorgen met name voor de productie en afgifte van geslachtssteroïden, o.a. testosteron en oestradiol, door de gonaden bij respectievelijk mannelijke en vrouwelijke dieren. Deze steroïden zijn van essentieel belang voor de productie van zaadcellen en eicellen. In de veterinaire en humane geneeskunde kan het wenselijk zijn om het hierboven genoemde proces langdurig te blokkeren om voortplanting en voortplantingsgedrag te voorkomen, of bij de behandeling van voortplantingsgerelateerde afwijkingen. Dit kan worden gedaan door middel van zogenaamde GnRH-analoga. Dit zijn op GnRH lijkende moleculen die synthetisch gemaakt worden. GnRH-analoga hebben echter een korte werkingsduur waardoor grote hoeveelheden moeten worden toegediend om tot een effectieve behandeling te komen. Dit maakt deze behandelmethode, in het bijzonder voor veterinaire toepassingen, te duur. Bovendien hebben GnRH-analoga vaak ongewenste bijwerkingen en werken ze niet in alle diersoorten.
Voor zowel humane als veterinaire toepassing zou een vaccin tegen GnRH een goedkoop, langdurig werkend en veilig alternatief kunnen vormen voor de behandeling met GnRH-analoga. Het ontwikkelen van een vaccin tegen GnRH wordt echter bemoeilijkt doordat GnRH een lichaamseigen en bovendien klein peptide molecuul is. Het is van zichzelf niet immunogeen, en dus wordt er geen immuunreactie opgewekt. De meest toegepaste manier om een peptide immunogeen te maken, is het koppelen van het peptide aan een groot lichaamsvreemd eiwit. Dit lichaamsvreemde complex, het antigeen, wordt vervolgens toegediend in een adjuvant, een stof die het immuunsysteem extra stimuleert. Hierdoor ontstaat een nog sterkere immuunreactie, waardoor grote hoeveelheden antilichamen in het bloed worden afgescheiden, die vervolgens het lichaamseigen GnRH neutraliseren. Aangezien de keus in adjuvantia beperkt is, omdat ze in veel gevallen ongewenste ontstekingsreacties op de plaats van de injectie veroorzaken, moet vooral de immunogeniciteit van het antigeen verbeterd worden om te komen tot een effectief GnRH-vaccin. De in dit proefschrift beschreven studies zijn uitgevoerd om te komen tot een sterk

immunogeen GnRH-antigeen dat de basis vormt voor een vaccin met verschillende toepassingen. In hoofdstuk 2 van dit proefschrift is een overzicht gegeven van de literatuur met betrekking tot vaccinatie tegen GnRH.

Immunogeniciteit en karakterisering

In hoofdstuk 3 wordt beschreven hoe de immunogeniciteit van het GnRH-peptide kan worden verbeterd door het peptide te verlengen met de eigen aminozuursequentie tot het GnRH-tandem peptide, het vervolgens te dimeriseren (GnRH-tandem-dimeer) en te voorzien van vreemde aminozuren (G6k-GnRH-tandem-dimeer). Het vaccin gebaseerd op dit gemodificeerde peptide gekoppeld aan het eiwit ovalbumine bleek zeer effectief de testisfunctie van mannelijke biggen te blokkeren.
In hoofdstuk 4 is de rol van de verschillende aminozuren in het G6k-GnRH-tandem-dimeer peptide onderzocht door ze één voor één te vervangen door het aminozuur alanine. Sommige aminozuren konden eenvoudig worden vervangen zonder dat dit ten koste ging van de de effectiviteit van het vaccin. Dit biedt de mogelijkheid om het G6k-GnRH-tandem-dimeer peptide nog lichaamsvreemder te maken en dus een verhoogde immuunreactie te induceren. Vervanging van een aantal andere aminozuren leidde niet tot het gewenste effect, wat aangeeft dat deze aminozuren essentieel zijn voor het opwekken van een effectieve immuunreactie tegen GnRH.
Recent zijn bij zoogdieren enkele isovormen van GnRH ontdekt die mogelijk processen reguleren welke niet gerelateerd zijn aan de voortplanting. Aangezien de functies van deze GnRH-isovormen nog niet geheel bekend zijn, moet worden voorkomen dat door GnRH-vaccinatie ook deze GnRH-isovormen worden geneutraliseerd. In hoofdstuk 5 is onderzocht in hoeverre antilichamen opgewekt tegen een aantal verschillende GnRH-tandem-dimeer peptiden, de verschillende GnRH-isovormen herkennen. Het merendeel van de GnRH-peptiden bleek antilichamen op te wekken die naast GnRH, ook de isovormen herkenden. Twee peptiden wekten GnRH-specifiek antilichamen op, deze herkenden wel GnRH, maar niet de isovormen.

Toepassingsmogelijkheden

Het GnRH-vaccin zou toegepast kunnen worden als alternatief voor castratie van mannelijke biggen. Wereldwijd worden jaarlijks honderden miljoenen mannelijke biggen onverdoofd gecastreerd gedurende de eerste weken van hun leven, om zo te voorkomen dat het vlees van deze varkens de zogenaamde berengeur gaat vertonen. Deze onaangename geur kan worden waargenomen bij de bereiding en consumptie van het vlees en wordt veroorzaakt door de stoffen androstenon en skatol in het vetweefsel. Androstenon wordt in de testes geproduceerd en skatol is een afbraakprodukt van eiwitten uit het voer. De concentratie van beide stoffen in het vetweefsel is afhankelijk van de activiteit van de

testes. Alhoewel castratie de aanwezigheid van deze stoffen in het vetweefsel voorkomt, is het een dieronvriendelijke methode, welke pijn en stress veroorzaakt en bovendien kan leiden tot een verhoogde gevoeligheid voor ziekten. In de afgelopen decennia is gezocht naar alternatieven voor onverdoofde castratie, maar deze bleken tot op heden niet haalbaar of niet bruikbaar in de praktijk. Vaccinatie tegen GnRH vormt een goed alternatief; de door het vaccin opgewekte antilichamen neutraliseren GnRH, waardoor de afgifte van LH wordt geremd. Door de sterke daling in LH-afgifte worden er in de testes geen geslachtshormonen meer geproduceerd, waaronder testosteron en androstenon. Als gevolg hiervan is het vlees vrij van berengeur.

Toepassing van een GnRH-vaccin bij varkens vereist een methode om het gebruik en de werking van het vaccin te kunnen controleren. Dit is mogelijk door het in hoofdstuk 6 beschreven vaccinatieschema toe te passen. Hierbij worden de dieren op een leeftijd van ongeveer 9 en 17 weken gevaccineerd en 5 weken na de tweede vaccinatie geslacht. Effectief gevaccineerde dieren blijken duidelijk kleinere testes te hebben dan onbehandelde dieren, hetgeen een garantie vormt voor de afwezigheid van berengeur in het vlees.

Intacte mannelijke varkens groeien efficiënter dan gecastreerde varkens door de anabole werking van de mannelijke geslachtssteroïden. De groei van de varkens die behandeld zijn met het GnRH-vaccin blijkt te worden bepaald door de effectiviteit van de immuunreactie na de vaccinaties. Gevaccineerde varkens die direct al op de eerste vaccinatie reageren met een effectieve immuunreactie (waargenomen aan de hand van gereduceerde LH- en testosteronniveaus) vertonen een even efficiënte groei als gecastreerde varkens. Wordt de effectieve immuunreactie pas na de tweede vaccinatie waargenomen, dan vertonen deze dieren een efficiëntere groei dan gecastreerde varkens en een karkaskwaliteit die gelijk is aan die van onbehandelde mannelijke varkens.

GnRH-vaccinatie zou ook bij andere diersoorten als een alternatief voor castratie kunnen worden toegepast. In hoofdstuk 7 is de toepassing in mannelijke pony's beschreven. Om de kans op bijwerkingen rond de injectieplaats zoveel mogelijk te voorkomen, zijn vaccins met de milde adjuvantia CoVaccine en Carbopol getest. Beide vaccins werden goed verdragen, er traden nauwelijks bijwerkingen op als gevolg van de vaccinaties. In alle vier pony's gevaccineerd met GnRH-CoVaccine werd een sterke immuunreactie waargenomen na de tweede vaccinatie. Dit resulteerde in een abrupte daling van de testosteronconcentraties in het bloed. Als gevolg hiervan werd er een effect op de testes waargenomen; de diameter van de zaadbuisjes nam af, er trad atrofie op van de geslachtshormoon producerende Leydig cellen en er werd een afname in de bewegelijkheid van het sperma waargenomen. In de GnRH-Carbopol groep reageerde slechts één van de vier dieren op de vaccinaties met een tijdelijke afname van de testosteronconcentraties. Geconcludeerd kan worden dat GnRH-CoVaccine effectiever is in het blokkeren van de testosteronproductie dan GnRH-

Carbopol. In deze studie is niet onderzocht of GnRH-vaccinatie ook tot onvruchtbaarheid leidt, wel was bij enkele dieren het libido verminderd.
In de humane geneeskunde zou GnRH-vaccinatie kunnen dienen als vervanging voor hormoontherapie bij behandeling van prostaatkanker. Hormoontherapie bestaat o.a uit het toedienen van GnRH-analoga, die de productie van androgenen door de testes onderdrukken. Androgenen fungeren als groeifactor voor de tumoren. Gezien de bijwerkingen en de kosten van deze therapie, zou GnRH-vaccinatie een goed alternatief kunnen vormen. Voordat het vaccin in prostaatkankerpatiënten getest kan worden moeten er eerst veiligheidsstudies in proefdieren worden uitgevoerd. In hoofdstuk 8 zijn de resultaten beschreven van een studie in varkens, waarbij twee potentiële vaccins met verschillende adjuvantia zijn getest, te weten met ISA 51 en met CoVaccine. Beide vaccins zijn toegediend met hoge doseringen antigeen. GnRH-CoVaccine bleek effectiever dan GnRH-ISA 51; behandeling met GnRH-CoVaccine onderdrukte de testosteronproductie in alle 30 gevaccineerde dieren, in de GnRH-ISA 51 groep gebeurde dit bij 18 van de 30 dieren. Zowel op korte als op lange termijn werden geen afwijkingen waargenomen in de gezondheid van de dieren, beoordeeld aan de hand van klinische observatie, bloed - en urinebeeld en beoordeling van de organen.
In hoofdstuk 9 zijn de resultaten van de beschreven studies samengevat en de toepassingsmogelijkheden bediscussieerd.

Conclusie
Concluderend kan worden gesteld dat het geoptimaliseerde GnRH-antigeen de basis kan vormen voor een effectief GnRH-vaccin met naar alle waarschijnlijkheid vele toepassingsmogelijkheden. Voor veterinaire toepassing is het een alternatief voor onverdoofde castratie van biggen, wat zou moeten leiden tot afschaffing van deze dieronvriendelijke praktijken en wat tevens leidt tot betere groeiprestaties. In de humane geneeskunde kan GnRH-vaccinatie dienen als vervanging van hormoontherapieën bij ziekten die worden gestimuleerd door geslachtshormonen. Met name bij prostaatkanker, waar grote hoeveelheden hormonen moeten worden toegediend, de therapie bijwerkingen geeft en duur is, kan GnRH-vaccinatie een aantrekkelijk alternatief vormen.

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Curriculum Vitae

Johan Turkstra is geboren op 6 januari 1966 in Ommen en groeide op in Dwingeloo. In 1984 behaalde hij zijn HAVO diploma aan het Menso Alting College in Hoogeveen en begon in datzelfde jaar een opleiding aan de Christelijke Agrarische Hogeschool in Dronten. Na het behalen van het diploma in de richting Veehouderij (1988), heeft hij de studie Zoötechniek gevolgd aan de Landbouw Universiteit in Wageningen. Van 1992 tot 2001 werkte hij bij het Centraal Diergeneeskundig Instituut (later Instituut voor Dierhouderij en Diergezondheid) in Lelystad op de afdeling Moleculaire Herkenning onder leiding van Prof. Dr. R.H. Meloen aan het ontwikkelen van een vaccin ter voorkoming van berengeur bij mannelijke varkens. Sinds 2001, toen deze afdeling verzelfstandigd verder ging onder de naam Pepscan Systems, werkt hij bij dit bedrijf aan het ontwikkelen van vaccins tegen groeifactoren die betrokken zijn bij tumorgroei.