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Effects of GnRH immunization in sexually mature pony stallions

J.A. Turkstra[a,*], F.J.U.M. van der Meer[b], J. Knaap[c], P.J.M. Rottier[b], K.J. Teerds[d], B. Colenbrander[e], R.H. Meloen[a]

[a] *Pepscan Systems, Edelhertweg 15, 8219 PH Lelystad, The Netherlands*
[b] *Department Immunology and Infectious Diseases, Division of Virology, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 1, 3584 CL Utrecht, The Netherlands*
[c] *Research Institute for Animal Husbandry, Runderweg 6, 8219 NL Lelystad, The Netherlands*
[d] *Department Animal Sciences, Human and Animal Physiology Group, Wageningen University, Wageningen, The Netherlands*
[e] *Department Equine Science, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 1, 3584 CL Utrecht, The Netherlands*

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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 three 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.

* Corresponding author. Tel.: +31 320 237212; fax: +31 320 238120.
E-mail address: j.turkstra@pepscan.nl (J.A. Turkstra).

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Semen evaluation showed that from 2 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 three out of four 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™ 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.

Keywords: GnRH immunization; Horse stallions; Testosterone; Testis function

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 6 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 commerciallly 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 vaccines 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 3 weeks before the start of the experiment by removing the left testis, were assigned to three groups (GnRH/CoVaccine, GnRH/Carbopol and controls) of four 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 milliliter 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 3 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 2 weeks by puncturing of the jugular vein for determination of GnRH antibody titers and testosterone concentrations. Serum was prepared by centrifugation at 1400 g for 15 min. 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 and Colenbrander (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%

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 organized 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

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 35–70 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.

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 ± S.D. 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 to 76% binding of iodinated GnRH in a RIA, in the stallions treated with GnRH/CoVaccine (Fig. 1). Antibody titers remained at a high level in this group, resulting in undetectable testosterone levels 2 weeks after the second vaccination. Testosterone levels remained undetectable until the end of the experiment (Fig. 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.

The deprivation of testosterone was reflected in the quality of the semen (Table 2). Within 4 weeks after the second vaccination, sperm motility was reduced in all four stallions of the

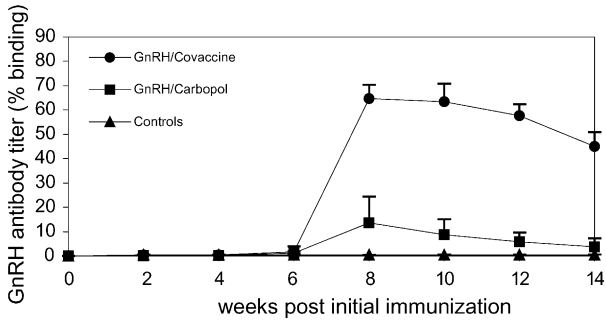

Fig. 1. Mean antibody titers against GnRH measured with a radio-immuno-assay and presented as the percentage of iodinated GnRH bound by a 1:2000 final serum dilution of stallions injected, at day 0 and 6 weeks later, with the G6k-GnRH-tandem-dimer-OVA conjugate in CoVaccine^{TM} HT adjuvant (GnRH/CoVaccine),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

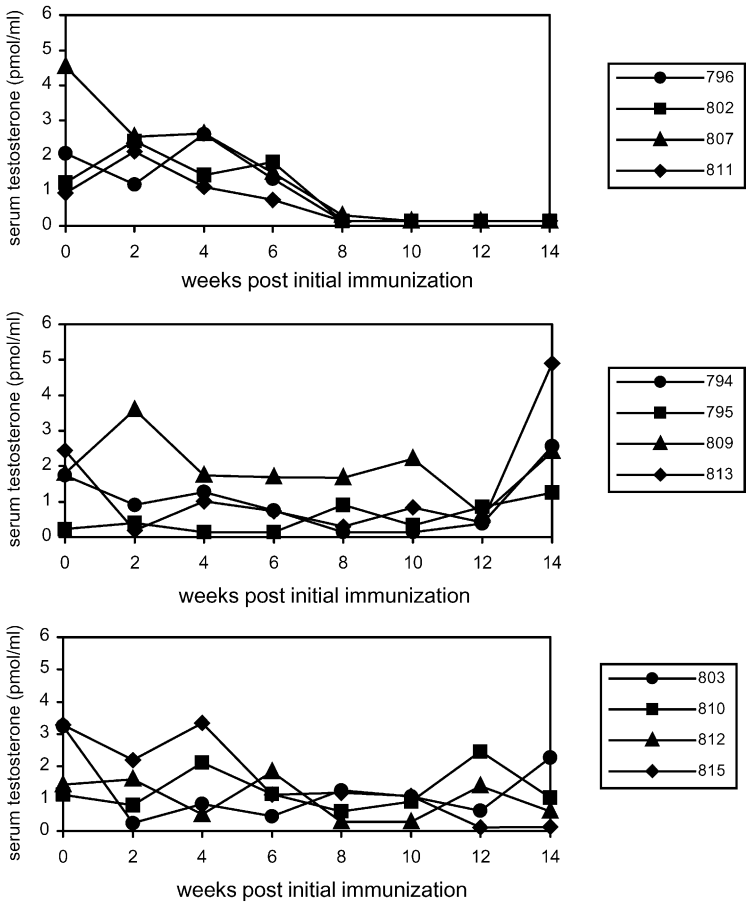

Fig. 2. Serum testosterone concentrations of stallions injected, at day 0 and 6 weeks later, with GnRH/CoVaccine (upper panel), GnRH/Carbopol (middle panel) and with the control emulsion (lower panel).

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

Table 2
Sperm characteristics of pony stallions immunized at week 0 and 6 with G6k-GnRH-tandem-dimer-OVA conjugate in CoVaccine™ HT adjuvant (GnRH/CoVaccine) or Carbopol (GnRH/Carbopol) and of control stallions

Animal number	Semen volume (ml)		Sperm motility (%)		Sperm concentration ($\times 10^6$/ml)		Total number of sperm ($\times 10^6$)	
	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	15	−3.0[a]	35[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	−0.4	51	−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	21	0.5b	71[b]	−3.8	204[b]	−75	3725

Values are expressed as the slope of values of weeks 0, 2, 4, 6, 8, 10 and 13 (slope) and as 'post booster values', the mean value of weeks 8, 10, 13 (weeks 8–13). Means within a column with a different letter differ ($P < 0.05$).
* Values of week 13 are not included, because no ejaculate could be collected at that time due to suppressed libido.

(Fig. 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.

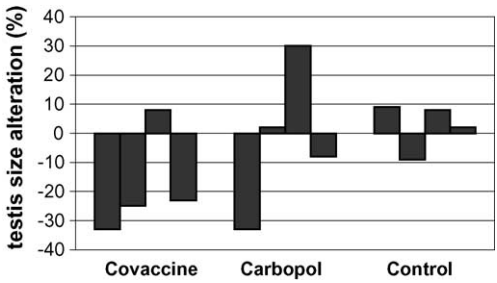

Fig. 3. Alterations in mean testis lenght during the experimental period for individual stallions treated with GnRH/CoVaccine (numbers 796, 802, 807, 811), GnRH/Carbopol (numbers 794, 795, 809, 813) and the control emulsion (numbers 803, 810, 812, 815).

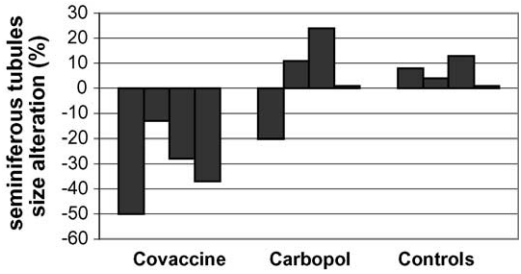

Fig. 4. Alterations in mean seminiferous tubuli diameter during the experimental period for individual stallions treated with GnRH/CoVaccine (numbers 796, 802, 807, 811), GnRH/Carbopol (numbers 794, 795, 809, 813) and with the control emulsion (numbers 803, 810, 812, 815).

Evaluation of the diameter of the seminiferous tubules demonstrated that the mean diameter at the end of the experiment was reduced by 10–50% 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 (Fig. 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 (Fig. 5). The mean Johnson score was significantly lower in both, the GnRH/CoVaccine and the GnRH/Carbopol group ($P = 0.01$). 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 (Fig. 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

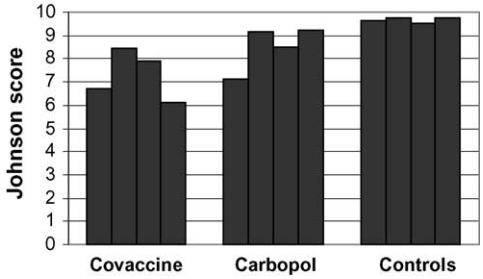

Fig. 5. Johnson score for the individual stallions treated with GnRH/CoVaccine (numbers 796, 802, 807, 811), GnRH/Carbopol (numbers 794, 795, 809, 813) and with the control emulsion (numbers 803, 810, 812, 815).

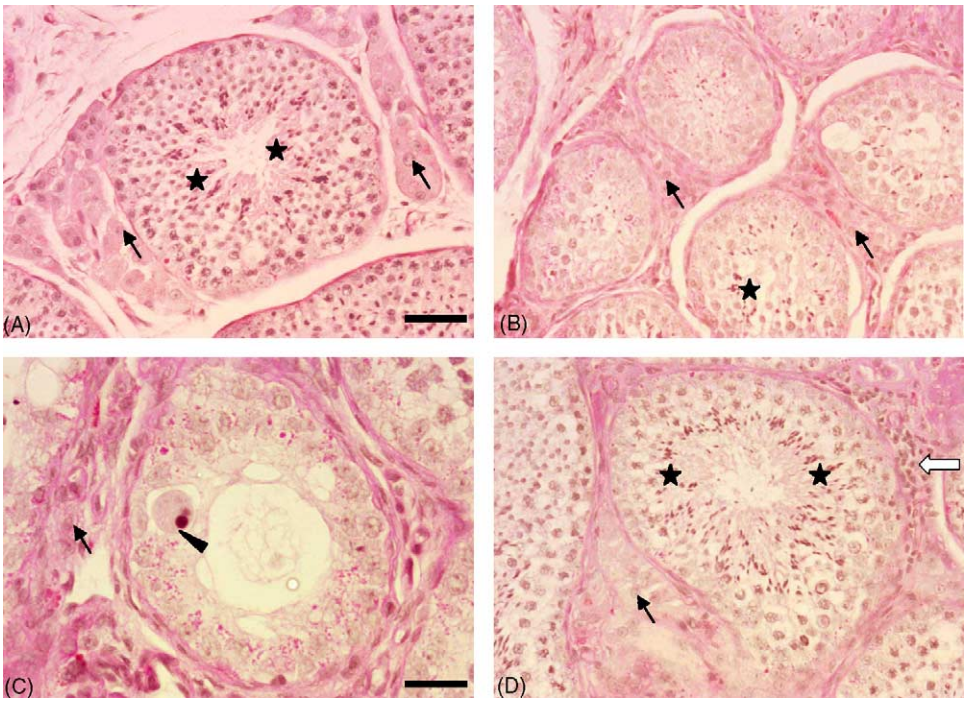

Fig. 6. (A) Cross-section of the testis of stallion 803 (control). Spermatogenesis in normal, many layers of different generation of germ cells are present, such as elongating spermatids, as well as a tubular lumen. In the interstitium Leydig cells can be easily recognized by the presence of a large round nucleus and one or two nucleoli. (B) Cross-section of the testis of stallion 811 immunized with GnRH/CoVaccine. In the seminiferous tubules the lumen is often absent and the tubular diameter has decreased compared to (A). In many tubules 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 $\times$235, bar 54 μm), while the magnification of figure C is $\times$475 (bar μm).

GnRH/Carbopol group, except for horse 794, a considerable number of tubules had no or a reduced lumen and spermatogenesis was slightly disorganized (Fig. 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 (Fig. 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 three animals of this group Leydig cell morphology was more or less normal (Fig. 6D) and similar to Leydig cells of control stallions (Fig. 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 2-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 two out of three treated stallions, though five 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 2 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 2 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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