Suppression of testicular function and sexual behavior by vaccination against GnRH (EquityTM) in the adult stallion

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Originally published at:
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Abstract

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Suppression of testicular function and sexual behavior by vaccination against GnRH (Equity™) in the adult stallion

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Abstract

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quality as well as sexual behavior. The inhibiting activity of Equity™ on these parameters is individually different and may last for a minimum of 6 months.

*Keywords*: Stallion; testosterone; sexual behavior; semen quality; GnRH; vaccine

### 1. Introduction

The suppression of male reproductive function has long been an important issue in equine veterinary practice. In stallions, the main interest is primarily directed towards preventing aggressive and unwanted sexual behavior to make them easier to handle and to guarantee their level of performance in training or competition. Control of reproductive activity may either be achieved by surgical, hormonal or immunological castration (Stout and Colenbrander, 2004; Stout 2005). Surgical castration which is widely used throughout the world irreversibly eliminates the source of testicular steroids and sperm resulting in loss of reproductive potential. Furthermore, castration carries surgical risks, especially in older animals and it has been shown (Line et al., 1985) that the behavioral problems will not be resolved in all cases. A non invasive alternative to castration is the application of progestagens to suppress hypothalamic and anterior pituitary secretion of GnRH and LH, respectively thus decreasing testicular testosterone release (Brady et al., 1997; Squires et al., 1997). Since progestagens such as altenogest must be given daily in high doses to control sexual behavior in older stallions (Miller at al., 1997) and because little is known about possible side effects, progestagen treatment has not been well accepted in practice. In addition, progestagen administration cannot be used in horses which are intended for human consumption and in many equine sporting events this treatment is considered as doping-offence.

Another possibility of reducing LH- and testosterone secretion in the stallion is the application of GnRH antagonists (Hinojosa et al., 2001; Fortier et al., 2002). The effect on libido appears to be age-dependent being absent in mature stallions despite a dramatic fall in testosterone concentrations (Fortier et al., 2002). Treatment with high doses of a GnRH agonist can effectively desensitize the pituitary to GnRH (down-regulation) in various species. In the stallion, however, agonist treatment had either no (Brinsko et al., 1998) or only a limited suppressive effect (Montavon et al., 1990; Boyle et al., 1991) and occasionally even enhanced gonadotropin secretion (Roser and Hughes, 1991; Sieme et al., 2004). While results using hormones, antagonists or agonists are not satisfactory, a practical alternative for inhibiting male reproductive function is by active immunization against various hormones of
the hypothalamic-pituitary-gonadal axis (D’Occhio, 1993; Thompson, 2000). Experiments using different GnRH vaccines were first performed in cattle (Robertson et al., 1979, 1982), sheep (Lincoln and Frazer, 1979; Jeffcoate et al., 1982) and swine (Falvo et al., 1986; Caraty and Bonneau, 1986). In horses, Schanbacher and Pratt (1985) were the first to describe the successful vaccination against GnRH in a cryptorchid stallion. Until now, several studies have been published (Dowsett et al., 1991, 1996; Malmgren et al., 2001; Dalin et al., 2002; Clement et al., 2005; Turkstra et al., 2005; Imboden et al., 2006; Elhay et al., 2007) not only investigating the anti-GnRH effect on reproductive activity in the horse but also for the treatment of equine arteritis virus (EVA) shedder stallions (Burger et al., 2004, 2006). In 2001, a GnRH vaccine (Equity™, Pfizer Animal Health, Australia) for specific use in horses was licensed in Australia and New Zealand for the control of estrus in the mare. As comprehensive studies in the stallion are lacking, the aim of the present investigation was to evaluate the safety and efficacy of Equity™ in the stallion with special emphasis on plasma testosterone and antibody concentrations as well as on semen quality and sexual behavior.

2. Material and methods

2.1. Experimental design

Eight clinically healthy and sexually experienced warmblood stallions from the National Stud in Avenches (Switzerland) aged between 6 and 15 years were used for the experiment. The animals were kept in box stalls bedded with straw and were fed hay or haylage, oats, barley, corn and pellets supplemented with minerals. Water was available at libitum. All animals were regularly exercised and had daily access to a paddock. Before the onset of the experiment the stallions were trained to mount the phantom and extragonadal sperm reserves were minimized by five daily semen collections (Hurtgen, 1992). Five stallions (A-E) were randomly allocated to a treatment group and 3 animals served as controls (F, G, H). Beginning in May collections of blood and semen were performed weekly for a period of one year. Scrotal width was measured using a calliper at the beginning of the experiment (May) and 4 (September), 8 (January) as well as 12 months (May) later. Subsequent to semen collections in weeks 1, 5 and 13 the stallions A-E were immunized with 1 ml (200 µg GnRH-protein conjugate) of a commercially available anti-GnRH vaccine (Equity™, Pfizer Animal Health, Australia). Control animals received an equivalent volume of saline solution. All injections were applied intramuscularly into the neck. Adverse effects were monitored for 3 days after injection by twice daily clinical examination with particular regard to body temperature, signs of tissue reaction at the site of injection and stiffness of the neck. All
animal experimentation was performed following approval from the local Animal Ethics Committee (Etat de Vaud, service vétérinaire, protocol approval number 1498).

2.2. **Semen collection and examination**

Once the stallion had mounted the phantom, semen was collected with an artificial vagina (model “Avenches”). Volume of the ejaculate was measured after removal of the gel fraction. Sperm concentration, total sperm number and total motility were assessed in freshly diluted (INRA 96, IMV, Aigle, France) semen with a sperm analyzer (HTM-IVOS, Version 12, Beverly, MA, USA) using 20 micron standard count analysis chambers (Art. no. SC 20-01-C, Leja, Nieuw-Vennep, The Netherlands) and standardized threshold values for stallion semen. For morphological examination, three drops of fresh semen were fixed in 2 ml buffered formol saline solution (Na$_2$HPO$_4$ 4.93 g, KH$_2$PO$_4$ 2.54 g, 38% formaldehyde 125 ml, NaCl 5.41 g, distilled water qs 1000 ml) and smears prepared. At least 200 spermatozoa were subsequently evaluated by phase contrast microscopy (Olympus BX50, UplanF1 100x/1.30) and abnormal spermatozoa classified in major (acrosome defects, nuclear vacuoles, abnormal heads, loose normal heads, abnormal midpieces, proximal droplets) and minor (loose normal heads, abnormal tails, distal droplets) defects (Blom, 1973; Jasko et al., 1990).

2.3. **Sexual behavior**

The stallions were exposed to the phantom in presence of an ovariectomized mare during maximal 10 min for each attempt to collect semen. Sexual behavior was measured as time to first erection and ejaculation.

2.4. **Testosterone analysis and GnRH antibody titers**

Blood samples (EDTA) were collected in the morning between 8 and 9 a.m. at each examination day by jugular venipuncture, immediately centrifuged (4000 x g, 10 min) and the plasma frozen (-20°C) until analysis. Testosterone was determined by electrochemiluminescence immunoassay (Elecsys 2010, Roche Diagnostics, Basel, Switzerland) as described earlier (Wang et al., 2004). The detection limit of the assay was 0.02 ng/ml. All samples were analyzed using a biotinylated monoclonal antibody against testosterone. Cross-reactivity with estrogens and progesterone was < 0.01%, with androstendione 0.91% and with 5-α-dihydrotestosterone 1.89%. For validation of between assay precision a pooled sample was analyzed 6 times a day for a total of 10 days (n = 60) and for within assay precision pooled samples were measured 20 times a day (n = 20). Inter- and intraassay coefficients of variation were 2.2% and 1.4%, respectively.
Antibody titers against GnRH were determined using a radioligand binding assay as described previously (Finnerty, 1994). Results are presented as percentage of total $^{125}$I-labelled GnRH bound at a plasma dilution of 1:40.

2.5. Data analysis

Data were analyzed using StatView 5.0 software program (SAS Institute, Wangen, Switzerland). A multivariate analysis of variance (ANOVA) was carried out to assess the effects of group allocation, time and vaccination (interaction between group allocation and time) on plasma testosterone concentrations and scrotal width. Between group differences were compared with unpaired $t$-test. Values were considered to be statistically significant at $P < 0.05$. Because of high individual variation antibody titers, plasma testosterone as well as total sperm number, motility and major sperm defects are shown for single animals. Testicular function was considered to be suppressed when testosterone values were lower than 0.1 ng/ml for at least 2 consecutive weeks. After a period of testicular suppression stallions were judged as having resumed reproductive activity when they fulfilled the following criteria, a) plasma testosterone concentrations > 0.1 ng/ml for at least 2 consecutive weeks, b) ejaculation of more than 500 million motile sperm within 10 min of exposure to the phantom and the ovariectomized mare.

3. Results

3.1. Adverse effects of the vaccine

In the 5 stallions immunized with 1 ml Equity™ neither apathy nor pyrexia could be diagnosed following the first or booster vaccinations. One animal (B) reacted with a small not painful swelling after the first and third immunization and another stallion (D) had a sore swelling after the third injection. All adverse effects resolved within 2-3 days after appearance. There were no injection site reactions in the control stallions.

3.2. GnRH antibody titer

Antibody titers against GnRH in all vaccinated stallions are shown in Fig. 1. Titers started to rise immediately after the second vaccination and reached peak values between 14.6% and 43.9% binding 2-4 weeks after the third vaccination. Thereafter the titers slowly decreased to values between 6.1% and 21.0% binding at week 51. The least antibody response with maximum values of only 15% binding occurred in stallion C.
Fig. 1. Anti-GnRH antibody titers (% binding at a 1:40 plasma dilution) in 5 stallions (A-E) after vaccination with Equity™ (↓).

3.3. Testosterone

Results from ANOVA demonstrate that weekly measured plasma testosterone concentrations were significantly influenced by group allocation ($P < 0.0001$), time of blood collection ($P < 0.0001$) and by vaccination (interaction between group and time, $P = 0.0003$). The individual testosterone profiles in all vaccinated stallions are shown in Fig. 2a. Starting at 2 weeks after the second vaccination only low fluctuating concentrations (< 0.1-0.5 ng/ml plasma) were observed in 4 stallions. In one animal (C) the range of values was higher (< 0.1-1.2 ng/ml plasma) and testosterone not suppressed during the whole study. Regarding duration of suppression 2 stallions had low plasma testosterone (< 0.1 ng/ml) during 45 weeks (B, 7-52) and 46 weeks (D, 6-52) while in the other two animals suppression lasted for 24 weeks (A, 7-31) and 36 weeks (E, 7-43), respectively. In control animals (Fig. 2b) testosterone secretion was episodic with fluctuations between 0.1 and 1.4 ng/ml plasma.
Fig. 2a. Individual blood testosterone concentrations of 5 stallions (A-E) after vaccination with Equity™ (↓).
Sexual behavior

In comparison to control stallions exhibiting normal sexual behavior including erection within 1-3 minutes and ejaculation during the first mount in the presence of a mare, libido varied considerably in all vaccinated stallions. In all but one stallion (D) time to erection and ejaculation was negatively affected by GnRH vaccination and in 2 stallions (A and C) both parameters were temporarily (weeks 20-42) prolonged and occasionally no semen could be collected. During this period stallion A often needed several mounts until ejaculation and stallion C failed to ejaculate at all during weeks 26-42. However, in these two animals libido normalized towards the end of the experiment. In the remaining 2 stallions the interest for mares continuously decreased after the second vaccination and no ejaculation occurred in
stallion B from week 29 until the end of the study (week 52) and in stallion E during weeks 12-42. In control animals semen collection was possible during the whole study.

3.5. **Semen quality**

In all vaccinated stallions total sperm number fluctuated between 2 and 18 x 10^9 and decreased after the third vaccination (Fig. 3a). In control animals total sperm number varied between 2 and 17 x 10^9 throughout the whole experiment (Fig. 3b). With the exception of stallion C sperm motility began to decrease individually after the second and third vaccination and recovered to prevaccination values after week 40 (Fig. 4a). In control animals sperm motility fluctuated on an individually different but constant level during the whole study (Fig. 4b). Changes in the percentage of major sperm defects in vaccinated stallions are shown in Fig. 5a. After immunization, an increase of major sperm defects was noticed in 3 stallions (A, B, D), while in stallion C a high percentage of major sperm defects (80-90%) was already present before the experiment started and these values were not affected by vaccination with Equity™. All control animals showed a high percentage of major sperm defects with only minor fluctuations on a constant level.

3.6. **Scrotal width**

Scrotal width was significantly \( P < 0.05 \) influenced by vaccination against GnRH (interaction between time and group allocation, \( P = 0.0073 \)) but not by group allocation (\( P = 0.1061 \)) and time (\( P = 0.0515 \)). Means \( (m \pm S.E.M.) \) of scrotal width in vaccinated animals decreased from 11.8 ± 0.6 cm before to 8.8 ± 1.0 cm and 8.0 ± 0.8 cm and increased to 10.6 ± 0.5 cm, 4, 8 and 12 months after first vaccination, respectively (Fig. 6). In control stallions mean scrotal width ranged between 11.0 ± 0.6 cm and 11.7 ± 0.9 cm. A significant \( P < 0.05 \) group difference was present 8 months after first vaccination.

3.7. **Resumption of reproductive activity**

Based on criteria defined in Material and methods only two vaccinated stallions A and E regained reproductive activity at weeks 34 and 43, respectively. In stallions B and D testicular function remained suppressed until the end of the study.
Fig. 3a. Total sperm number in weekly collected ejaculates of 5 stallions (A-E) after vaccination with Equity™ (↓).
Fig. 3b. Total sperm number in weekly collected ejaculates of the control stallions F, G and H.
Fig. 4a. Total sperm motility in weekly collected ejaculates of 5 stallions (A-E) after vaccination with Equity™ (↓).
Fig. 4b. Total perm motility in weekly collected ejaculates of the control stallions F, G and H.
Fig. 5a. Major sperm defects (%) in weekly collected ejaculates of 5 stallions (A-E) after vaccination with Equity™ (↓).
Months before and after the first vaccination

Fig. 5b. Major sperm defects (%) in weekly collected ejaculates of the control stallions F, G and H.

Fig 6. Scrotal width ($m \pm S.E.M.$) in 5 vaccinated (■) and 3 control (□) stallions before, 4, 8 and 12 months after the first vaccination with Equity$^{TM}$. *Significant difference between groups ($P < 0.05$, $t$-test).
4. Discussion

Results of our study demonstrate that administration of the GnRH vaccine Equity™ to adult stallions reduces testosterone secretion, scrotal width as well as semen quality and sexual behavior. The immune response to vaccination, however, was characterized by considerable variation as depicted in the profiles of the individual animals.

Unwanted side effects including minor swelling at the injection site lasting for only 2 to 3 days were observed in 2 stallions. The high degree of safety when using Equity™ in horses agrees with a recently published study in mares (Elhay et al., 2007) showing that only 10 of 24 vaccinated mares reacted with a transient slightly raised and flat swelling of the skin following first or second immunization. In contrast, the application of Improvac™ (Imboden et al., 2006) or other GnRH vaccine formulations (Dowsett et al., 1991, 1996; Dalin et al., 2002) in the horse may cause severe adverse effects as pyrexia, apathy and stiffness of the neck and should therefore not be used in horses.

First successful vaccination of a cryptorchid stallion against GnRH was reported by Schanbacher and Pratt (1985), but several booster injections were required to suppress testosterone secretion. This vaccine contained Freund's adjuvant and inhibition of testosterone lasted 4 months. Because of severe reactions at the site of injection other vaccine formulations containing a water-soluble adjuvant were subsequently developed. Using such a vaccine in 2-year-old colts (Dowsett et al. 1996) testicular function as well as sexual behavior and libido were individually suppressed for 12 to 26 weeks after two to three immunizations. In our study using the commercially available horse specific vaccine (Equity™) given three times, testosterone concentrations were suppressed (< 0.1 ng/ml) for a minimum of 24 weeks in 4 of 5 adult stallions. In 2 animals (B, D) testosterone remained low for 45 and 46 weeks, respectively. One stallion (C) showed a very poor rise in antibody titer without any visible effect on testosterone secretion. This clearly shows that the application of Equity™ in adult stallions may elicit variable immune responses with different antibody titers and different duration of testosterone suppression.

A negative influence of GnRH immunization on semen quality was observed in 4 of 5 stallions and the drop of total sperm number and sperm motility as well as the increase in major sperm defects varied considerably between single animals. These results agree with others studies (Dowsett et al., 1996; Malmgren et al., 2001; Clement et al., 2005; Turkstra et al., 2005) showing that good responders (high antibody titers) reacted with a reduced semen quality. In our experiment the most dramatic decrease in sperm motility from 79% to 3% within 6 months after the first vaccination occurred in stallion B and this animal also had a
marked increase in major sperm defects. Lower sperm production, lower sperm motility
and an increase in sperm abnormalities are in accordance with low testosterone concentrations
caused by high circulating antibody titers. These results confirm the essential role of
testosterone for spermatogenesis and epididymal function but leaves the question open why
the changes in semen quality were highly variable between individual stallions despite
constant low testosterone concentrations for more than 6 months. A possible explanation
might be the different effect of GnRH immunization on pituitary LH and FSH secretion
(Garza et al., 1986; Rabb et al., 1990). Because FSH is less affected by GnRH, circulating
FSH together with low testosterone concentrations may be sufficient to maintain
spermatogenesis.
Regarding sexual behavior the application of Equity™ reduced libido in 4 of 5 stallions.
Interestingly enough, in stallion C which had the lowest antibody titers and highest
testosterone concentrations, libido and mounting behavior were markedly reduced during
weeks 26-39. Quite the opposite situation was observed in stallion D which exhibited strong
sexual and mounting behavior during the whole experiment in spite of high antibody titers
and low testosterone values. From these findings it is obvious that sexual behavior is not only
testosterone dependent but may rather be influenced by various others factors as age of the
animal and previous sexual experience (Stout, 2005). Looking at other studies in which
GnRH vaccination had either no (Clement et al. 2005) or only a limited effect (Malmgren et
al., 2001; Turkstra et al., 2005) on libido, the use of Equity™ in our experiment was more
successful to control stallion behavior. However, because of differences in vaccine
formulations and dosage, number of boosters as well as age, breed and number of vaccinated
stallions comparison of results between these studies is difficult.

5. Conclusion
This study demonstrates that 3 immunizations with Equity™ are well tolerated and can
reliably suppress testicular function and sexual behavior in adult sexually experienced
stallions. The inhibitory effect, however, is highly variable and may last from a minimum of
24 weeks to more than 46 weeks.

Acknowledgements
We would like to thank G. Cosentino of the Laboratory Dr. Risch, Liebefeld, for the
testosterone analyses.
Conflicts of interest statement

None of the authors has any financial and personal relationships with other people or organizations that could inappropriate influence the study.

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