Reversibility of the effects of GnRH-vaccination used to suppress reproductive function in mares

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Summary

Reasons for performing study: Active immunisation against gonadotrophin-releasing hormone (GnRH) provides a reversible method for control of oestrous behaviour and fertility in mares. Previous reports failed to demonstrate the interval to resumption of cyclic ovarian activity after GnRH-vaccination.

Hypothesis: Administration of the GnRH-vaccine Improvac®[1] in a large group of mares of various ages will result in effective, reliably reversible suppression of ovarian activity within a two-year period.

Methods: The mares, subdivided into three age categories were vaccinated twice (with a 35 d interval) using Improvac®[1] and were monitored *via* blood samples until Day 720 after initial vaccination for serum progesterone concentration determination by radio-immune assay and anti-GnRH antibody titre by enzyme immuno-assay. Samples were collected until individuals resumed cyclic ovarian activity.

Results: All mares showed suppression of cyclic ovarian activity (SPC $\leq$1 nmol/l) and 92.2% resumed cyclic activity at Day 720 with a mean interval = 417.8 d (SD = 23.19) and median = 344 d. A significant age effect (P=0.028) on the interval, but not on GnRH-AB titre response, was observed between the youngest ($\leq$4 years) and oldest ($\geq$11 years) categories.

***Conclusions**:* Immunising adult mares of all ages with Improvac®[1] resulted in a reversible suppression of cyclic ovarian activity in most mares. An age effect, with the youngest mares showing a longer interval to reversibility was observed.

Introduction

Suppressing reproductive function and associated oestrous behaviour in mares has been managed by several approaches. Pharmacological methods utilising progesterone or progestins and potentially reversible GnRH-agonist or -antagonist therapy and their associated shortcomings including costs, practicality and potential negative side-effects are reported [1,2,3]. Surgical ovariectomy is generally considered to be undesirable, due to its associated risks, and irreversible loss of breeding potential [4].

Various immunocontraceptive methods have been used to control fertility in mares. Although pZP vaccine is effective, it neither prevents mares from cycling nor suppresses oestrus-related behaviours [5]. GnRH vaccines utilise a modified form of GnRH hormone conjugated to a foreign protein administered to stimulate the production of anti-GnRH antibodies. These antibodies neutralise endogenous GnRH, preventing natural binding to receptors on the pituitary gonadotrophs, suppressing pituitary secretion of follicle stimulating hormone (FSH) and luteinising hormone (LH) [6,7,8]. Studies report successful but variable suppression of ovarian activity [6,7,8,9,10,11,12,13]. Age effects on response and of using SPC to reliably monitor cyclic ovarian activity have been reported. [8,12]. Short monitoring periods generally limit these reports. Reliable reversibility and the associated duration of anoestrus induced by vaccination have not been reported.

This study investigated the reversibility of anoestrus over a period of two years in a large group of mares of different ages after two treatments with the GnRH-vaccine Improvac®[1].

Materials and methods

Experimental design

A previous controlled study reported on the efficacy, safety and interval to suppression of reproductive activity after vaccination of non-pregnant mares with Improvac®[1] [12]**.** The current study was designed as a continuation to observe the reversibility of treatment. Mares (n=51) aged between 3-17 years, and either Thoroughbred or similar breed, were subdivided into three age categories: Category 1 (≤4 years, n = 25), Category 2 (5-10 years, n = 16) and Category 3 (≥11 years, n = 10). The trial was approved by The University of Pretoria's Animal Use and Care Committee (V068/05).

GnRH immunization

The mares were injected into the gluteal muscles with 2 ml Improvac®[1] (400 µg RnRF-protein conjugate) approximately 3 months after the onset of the physiological breeding season in South Africa. All showed cyclic reproductive activity *via* trans-rectal palpation and ultrasound examination at the time of vaccination. One booster (2 ml) was administered 35 d after primary vaccination. By 70 days after primary vaccination, 100% of mares showed SPC reflecting anoestrus concentrations and ovarian activity was absent on clinical examination [12]. Resumption of ovarian activity was henceforth defined by a blood sample exceeding a

baseline SPC prospectively set at ≤6 nmol/l, the reported upper threshold concentration for winter anoestrus [11].

Blood sampling

Blood samples were collected by jugular venipuncture from Day 232 after primary vaccination at two-weekly intervals until resuming cyclic activity or failing that, until the end of the study on Day 720. The blood samples were collected in plain Vacutainer® tubes[3] and left to clot. Serum was separated by centrifugation (4000 x G, 10 min) on the same day before storage at -20ºC until assayed for both SPC and anti-GnRH antibody titre.

Serum progesterone assay

Assays for serum progesterone concentration (SPC) were conducted by means of radio-immune assay (RIA)[4]. Assay sensitivity was 0.06 nmol/l. The main cross-reactivity was with progesterone (100%), 5α-pregnane-3,20-dione (9.0%), 17α-hydroxyprogesterone (3.4%) and 5β-pregnane-3,20-dione (3.2%). The intra- and inter-assay coefficients of variation were 8.8% (low), 3.6% (medium), 2.7% (high) and 9.7% (low), 3.9% (medium and high), respectively.

GnRH-AB titre determination

The anti-GnRH titres were determined by enzyme immuno-assay (EIA), using a modification of the method described by Elhay *et al*. [11]. Briefly, 96 wells MaxiSorp MTPs[5] were coated overnight (ON) at 4°C with 10 µg/ml GnRH peptide in sodium carbonate buffer at pH 9.6. The plates were washed with phosphate-buffered saline (PBS) containing 0.05% Tween 20 and then blocked ON at 4°C with 0.3% BSA in PBS. Plates were incubated with serial dilutions (dilution factors 200, 400, 800, 1600, 3200, 6400, 12800) of test mare serum and

standards for 1 h at 37°C. The plates were washed and bound antibody was detected by incubating plates with protein G-horse radish peroxidise (HRP)[6] for 1 h at 37°C. Subsequently, plates were washed and bound protein G-HRP was visualised with tetramethylbenzidine microwell peroxidase substrate[7]. Pooled samples of high titre (high standard) at Day 70 and low titre (low standard) at Day 35 after primary vaccination and samples of unvaccinated horses (negative control) served as reference points for the unknown samples on all plates analysed.

The plates were normalized by dividing all optical densities (ODs) by the maximal OD, thereby setting the high standard in 1:200 dilution to 1.0 for each plate. Subsequently, a negative titre threshold was defined as OD of the average of the negative controls plus 2 standard deviations. In this regard, titres were determined as the first OD exceeding the negative titre (negative control) threshold (starting from dilution 1:12800 and decreasing). For method validation, titres of standards and positive controls were recorded for all 26 plates. The high and low standard titres were 3200 for all 26 plates and 800 for 18 plates and 400 for the remaining 8 plates, respectively. The negative control titres were 0 for all plates.

Statistical analysis

Data analysis was performed using Sigma Stat[8]. A Kruskal–Wallis one way analysis of variance (ANOVA) was performed on the ranked data to assess significance of age on the variables for general linear models. A Dunn's Multiple Comparison Test for means separation was also performed with Category 1 as control. Statistical significance was defined as $P<0.05$.

Results

Interval to resumption of cyclic activity

By Day 720, 47/51 (92.2%) mares had resumed cyclic activity with a mean interval of 417 d (SD 23.9) and median of 344 d. The remaining four mares (all Category 1) were still in anoestrus.

Effect of age on resumption of cyclic activity and GnRH-antibody titres

The median interval in Category 1 was significantly greater than in Category 3 mares on Day 330 (P=0.027) (n=21), and Day 720 (P=0.028) (Fig 1). The mean anti-GnRH-antibody titres of six mares randomly selected from each of Category 1 and 3 at 70 d post-primary vaccination and at six weeks prior to resuming cyclic activity showed no significant difference (Fig 2) although mean titres were higher at both intervals for Category 1 mares.

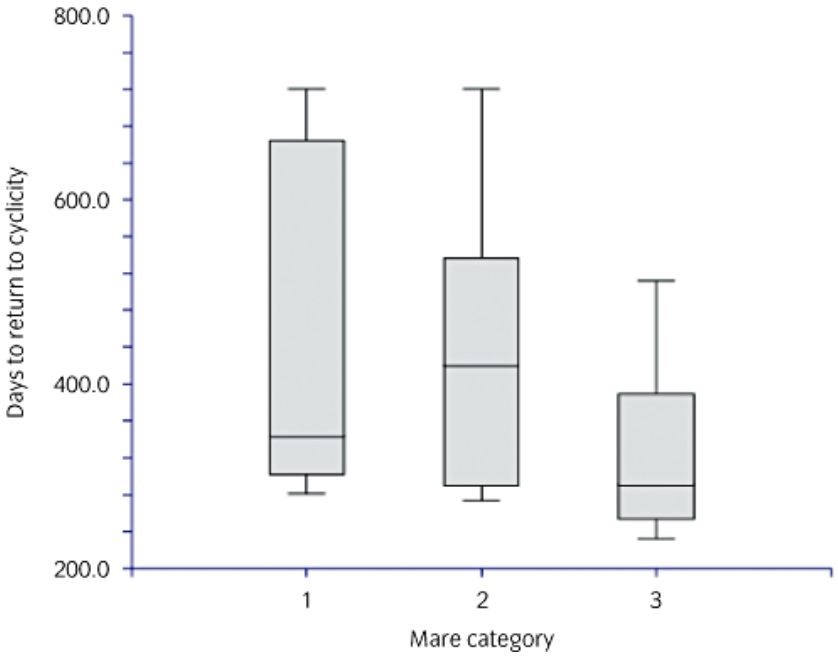

Fig 1. Box plot of days to return to cyclicity for different age categories of mares. Category 1 (≤4 years, n = 26), Category 2 (5-10 years, n = 18) and Category 3 (≥11 years, n = 11)

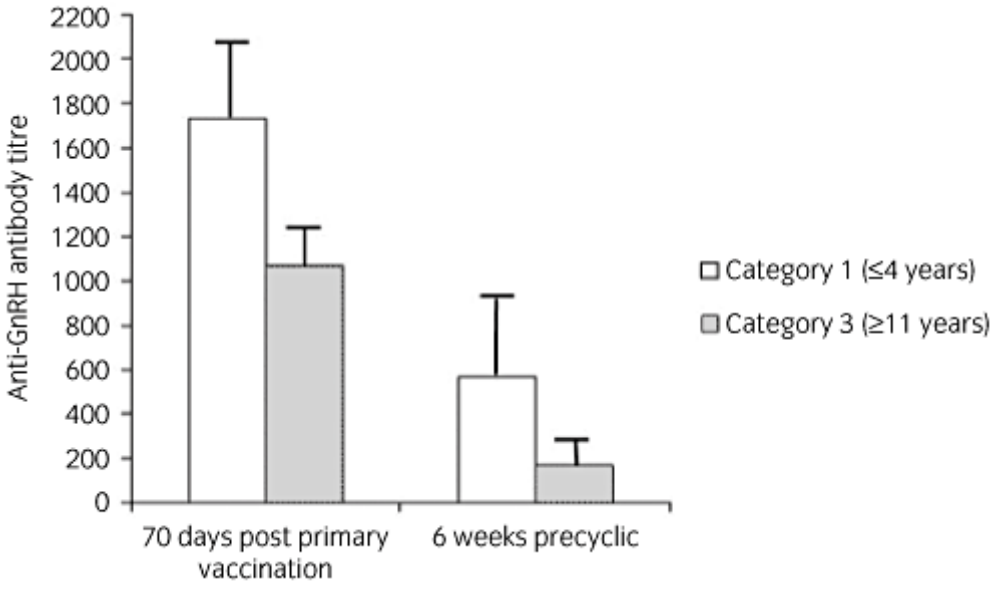

Fig 2. Mean (SEM) GnRH antibody titres for selected mares in Category 1 and 3 at 70 days post-primary vaccination and six weeks prior to resumption of cyclic activity

Discussion

In previous GnRH-vaccine studies, animal numbers were either small or monitoring discontinued before most had reversed. Dalin *et al.* (GnRH-bovine serum albumin-conjugate vaccine) treated three mares, two responded with reversal at 13.5 and 15 months after primary vaccination, respectively [8]. Imboden *et al.*, treating nine mares with Improvac®[1], reported down-regulation of ovarian activity, but not reversibility [10]. Elhay *et al.* reported using Equity®[2] in 24 mares [11]. No treated mare exceeded anoestrus SPC threshold levels at 20 weeks, and ovarian shutdown lasted 4-23 weeks in 10/16 mares. The other 6 mares remained inactive at the end of the 29-34 weeks observation period. Killian *et al.* comparing contraceptive methods, treated 15/43 mares with a single-shot GnRH-vaccine (GonaCon®[7]) [13]. The rates of contraception in these mares decreased from 94-40% between year 1-4, but time to resumption of cycling was undetermined.

The resumption of cyclic ovarian activity was used to monitor the reversibility of anoestrus induced in all mares following Improvac®[1] administration. By Day 720, all but 4 mares (all Category 1) had resumed ovarian activity with a median interval of 344 d. A significant effect of mare age on reversibility was found between the oldest and youngest categories. Stout and Colenbrander similarly reported two treatments of GnRH-tandem-dimer vaccine suppressed testosterone secretion in young sexually-mature stallions whereas older stallions generally required further boosters [2]. In this study, although anti-GnRH-AB titres were not significantly different between youngest and oldest mares, the mean titres were higher for the youngest and may explain their later return to cyclic activity.

In conclusion, variable reversal of anoestrus was shown in 92.2% of mares within two years after vaccination with Improvac®[1]. Older mares showed significantly earlier resumption of cyclic ovarian activity than the youngest mares.

Manufacturers' addresses

[1]Pfizer Animal Health, Sandton, South Africa

[2]Pfizer Animal Health, West Ryde, NSW, Australia

[3]BD Vacutainer Systems, Plymouth, PL6 7BP, UK

[4]Coat-a-Count, Diagnostic Products Corp, Los Angeles, CA, USA

[5]Nunc, Roskilde, Denmark

[6]Sigma-Aldrich, Steinheim, Germany

[7]KLP, Gaithersburg, USA

[8]Systat Software Inc., San Jose, CA, USA

References

[1] Skelton, K.V., Dowsett, K.F. and McMeniman, N.P. (1991) Ovarian activity in fillies treated with anabolic steroids prior to the onset of puberty. *J. Reprod. Fert. Suppl.* **44,** 351-356.

[2] Stout, T.A.E. and Colenbrander, B. (2004) Suppressing reproductive activity in horses using GnRH vaccines, antagonists or agonists. *Anim. Reprod Sci.* **82-83**, 633-643.

[3] Gee, E.K., DeLuca, C., Stylski, J.L. and McCue, P.M. (2009) Efficacy of Medroxyprogesterone Acetate in Suppression of Estrus in Cycling Mares. *J. Equine Vet. Sci.* **29**, 140-145.

[4] D'Occhio, M.J. (1993) Immunological suppression of reproductive functions in male and female mammals. *Anim. Reprod. Sci.* **33**, 345-372.

[5] Kirkpatrick, J.F., Rowan, A., Lamberski, N., Wallace, R., Frank, K. and Lyda, R. (2009) The practical side of immunocontraception: zona proteins and wildlife. *J. Reprod. Immunol.* **83,** 151-157.

[6] Garza, F., Thompson, D.L., French, D.D., Wiest, J.J., St George, R.L., Ashley, K.B., Jones, L.S., Mitchell, P.S. and McNeill, D.R. (1986) Active immunization of intact mares against gonadotropin-releasing hormone: differential effects on secretion of luteinizing hormone and follicle-stimulating hormone. *Biol Reprod.* **35**, 347-352.

[7] Tshewang, U., Dowsett, K.F., Knott, L.M. and Trigg, T.E. (1997) Preliminary study of ovarian activity in fillies treated with GnRH vaccine. *Aust. Vet. J.* **75,** 663 – 667.

[8] Dalin, A.M., Andresen, Ø. and Malmgren, L. (2002) Immunization against GnRH in mature mares: antibody titres, ovarian function, hormonal levels and oestrus behaviour. *J. Vet. Med.* **49**, 125-131.

[9] Stout, T.A.E., Turkstra, J.A., Meloen, R.H. and Colenbrander, B. (2003) The efficacy of GnRH vaccines in controlling reproductive function in horses. In: *Proceedings of an expert consultation on the Control of Wild Elephant Populations.* Utrecht University, Utrecht, pp 65-67.

[10] Imboden, I., Janett, F., Burger, D., Hässig, M. and Thun, R. (2006) Influence of immunization against GnRH on cycling activity and estrous behaviour in the mare. *Theriogenology* **66,** 1866-1875.

[11] Elhay, M., Newbold, A., Britton, A., Turley, P., Dowsett, K. and Walker, J. (2007) Suppression of behavioural and physiological oestrus in the mare by vaccination against GnRH. *Aust. Vet. J.* **85**, 39 – 45.

[12] Botha, A.E., Schulman, M.L., Bertschinger, H.J., Guthrie, A.J., Annandale, C.H. and Hughes, S.B. (2008) The use of a GnRH vaccine to suppress mare ovarian activity in a large group of mares under field conditions. *Wildl. Res.* **35**, 548-554.

[13] Killian, G., Thain, D., Diehl, N., Rhyan, J. and Miller, L. (2008) Four-year conception rates of mares with single-injection porcine zona pellucida and GnRH vaccines and intrauterine devices. *Wildl. Res.* **35**, 531-539.