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COMMUNICATION

Evaluation of prostaglandin $F_{2\alpha}$, estradiol benzoate and deslorelin acetate protocol for oestrus induction in bitches

Evaluación de un protocolo con prostaglandina $F_{2\alpha}$, benzoato de estradiol y acetato de deslorelina para la inducción de estro en perras

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RESUMEN

En perras, el intervalo interestro es relativamente más largo que en otras especies de animales domésticos, muchos esfuerzos se han hecho para reducirlo debido al incremento en el mercado de mascotas. El objetivo de este estudio fue probar la eficacia de un protocolo con prostaglandina $F_{2\alpha}$, benzoato de estradiol y acetato de deslorelina para la inducción del estro. Se utilizaron seis perras, dos estaban en el día 75 de anestro posparto, y cuatro estaban en diestro, a estas últimas se les indujo luteólisis con prostaglandina $F_{2\alpha}$ (2.5 µg/kg/24h/4d *sc*). Todas las perras recibieron una única dosis de benzoato de estradiol en el día 75 de anestro (posparto o inducido). Se les aplicó acetato de deslorelina diariamente desde el día 76, hasta que los niveles de progesterona séricos fueron entre 1,5 y 2,5 ng/ml. Se calculó la proporción de perras inducidas al proestro, de ovulación y de preñez, y se aplicó la prueba no paramétrica de suma de clasificación de Wilcoxon. Todas las perras entraron en proestro en los días 4-5. Todas las perras fueron inseminadas tres a cinco días luego de que los niveles de progesterona sérica estaban entre 1,5 y 2,5 ng/mL. Ninguna perra ovuló, ni quedó gestante. Se concluye que este protocolo es eficaz para inducir el estro, pero no para inducir la ovulación.

Palabras clave: análogo de GnRH, anestro, canino, sincronización.

SUMMARY

Inter-oestrus interval in bitches is relatively longer than in other domestic animal species, efforts have been brought about to reduce it due to the increase in the pet market. The aim of this study was to test the efficacy of a prostaglandin $F_{2\alpha}$, estradiol benzoate and deslorelin acetate protocol for oestrus induction in bitches. Six healthy female dogs were used. Two bitches were on day 75 of postpartum anoestrus, the other four dogs were in dioestrous and a luteolysis was induced in those ones with prostaglandin $F_{2\alpha}$ (2.5 µg/kg/24h/4d *sc*). All bitches received a single dose of estradiol benzoate on day 75 of anestrous (postpartum or induced). On day 76 and every day onwards, injection of deslorelin acetate was applied until the seric progesterone levels were between 1.5 and 2.5 ng/mL. The proportions of proestrous-induced bitches and of ovulating bitches as well as pregnancy rates were measured, and Wilcoxon Rank-Sum nonparametric statistical test was applied. All female dogs became in proestrous on day 4.5. All bitches were artificially inseminated three and five days after progesterone levels were between 1.5 and 2.5 ng/mL. No bitches ovulated and no pregnancy was achieved. We conclude that this protocol is effective to induce estrus, but not to induce ovulation.

Key words: anoestrus, canine, GnRH analogue, synchronization.

INTRODUCTION

Pet market is a growing worldwide business. To improve kennel efficiency, pharmacological manipulation for oestrus induction and ovulation has been used to shorten the bitch anoestrus period, which is particularly long (4-8 months) (Concannon *et al* 2009, Concannon 2011). Furthermore, estrus induction is indicated for primary and secondary anoestrus treatment, for basic research in reproductive physiology, and for the development of other biotechnologies such as embryo transfer (Kutzler 2005, Kutzler 2007, Root Kustritz 2012).

Several protocols to induce oestrus have been tested. Dopamine agonists such as cabergoline (5 µg/k/d) and bromocriptine (20 µg/k/d), have been the most frequently used, with oestrus induction rates between 80-100%. Gonadotropins and estrogens presented more variable oestrus induction rates (Walter *et al* 2011).

However, Stornelli *et al* (2012) achieved oestrus induction rates of 80% using one dose of equine chorionic gonadotropin (eCG) (50 IU/k), followed by one dose of human chorionic gonadotropin (hCG) (500 IU) seven days after. With estrogen, variable estrus induction rates have been achieved, reaching 100% using diethylstilbestrol (5 mg *per os* once daily for 6-9 days) (Kutzler 2007). The main disadvantage with the use of dopamine agonists is the uncertainty regarding the duration of the treatment that can be extended more than 40 days for estrus induction

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Table 1. Description of the experimental animals.
Descripción de los animales experimentales.

Animal	Breed	Age (years)	Oestrus cycle	Inter-estrus interval (days)	Anoestrous (days)
FPP1	French Bulldog	2	Postpartum anoestrus	223	70
FPP2	French Bulldog	3	Postpartum anoestrus	216	83
FD1	French Bulldog	3	Dioestrus	210	–
FD2	French Bulldog	3	Dioestrus	218	–
ED1	English Bulldog	1.5	Dioestrus	187	–
ED2	English Bulldog	2.5	Dioestrus	210	–

(Wiebe and Howard 2009, Root Kustritz 2012). Prolonged use of estrogen generates myelotoxicity and aplastic anemia (Weiss 2003, Sontas *et al* 2009, Schnelle and Barger 2012), and in many countries its use is prohibited. Prostaglandins by themselves, do not induce oestrus, and produce some unwanted side effects (vomiting, salivation, diarrhea, tremors and abdominal pain) (Eilts 2002).

Gonadotropin-releasing hormone (GnRH) agonists have successfully induced estrus in dogs, depending on the type of molecule and formulation (Concannon 2011, Root Kustritz 2012, Wiebe and Howard 2009). Deslorelin acetate (DA) is a non-peptide analogue of natural GnRH, used for estrus induction and ovulation in mares[1] (CMPVU 2009). In dogs, it has a 150 times greater power than endogenous GnRH (Gobello 2007), and when used in subcutaneous implants (2.1 to 4.7 mg) its efficacy in inducing oestrus in dogs is 100%. However, it has pregnancy rates ranging from 40 to 87%, and pregnany luteal failure has also been observed (Kutzler 2007, Walter *et al* 2011). It also presents the disadvantage of having to perform an incision in the skin to remove the implant. One study (Lanna *et al* 2010) using a slow-release injectable form of DA[2] achieved 100% of estrus induction, to 2 mg im/48 h four times, without producing unwanted side effects at the site of injection. Concannon *et al* (2006) have used another GnRH agonist (lutrelin) with the same power as DA, proposed a dose of 0.6 µg/k (0.2-1.2 µg/k) with an efficiency of 92% in oestrus induction and 100% pregnancy rate, suggesting that this same DA dose can be used, to obtain similar results for a oestrus induction protocol in dogs.

The aim of this study was to evaluate the effectiveness of a protocol based on sustained release of injectable DA, estradiol benzoate (EB) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) to induce oestrus, resolve the minimal actual length of anoestrus necessary for pregnancy, induce fertile estrus, induce adequate luteinisation and achieve pregnancy with a shortened interoestrous interval.

MATERIALS AND METHODS

ANIMALS

Six healthy bitches from different commercial kennels, English Bulldog (n = 2) and French Bulldog (n = 4), aged 1.5 to 3 years, with a body weight of 17.5 and 20 kg for the English Bulldog and 9.6±0.3 kg for the French Bulldog (all with body condition 6 on a score of 1-9) were used. All animals were individually housed in pens, at the CESVET Veterinary Hospital kennel and were fed a commercial diet twice daily and water *ad libitum*. The protocol was conducted under the stipulations of the Colombian National Animal Protection Statute (law 84 of 1989 of Colombian Government).

The oestrous cycle preceding the study had an inter-estrus interval between 187 and 223 days (210.7±12.6) and in previous cycles it was very similar, as determined based on the breading records. Two bitches were in postpartum anoestrus (70 and 83 days postpartum), with serum progesterone (sP4) levels < 0.5 ng/mL and a vaginal cytology (VC) profile of > 90% of small parabasal and intermediate cells; the remaining four dogs were on day 39±5.4 post-luteinising hormone (LH) peak of a spontaneous estrous cycle (sP4 > 5 ng/mL), during which they were not mated (table 1).

HORMONAL TREATMENT

To induce luteolysis, $PGF_{2\alpha}$[3] was administered for 4 days in a single *sc* injection of 2.5 µg/kg/d to diestrus dogs (39 ± 5.4 days post natural LH peak). sP4 was measured before and after treatment (figure 1). Anoestrus was confirmed by sP4 and VC in all bitches on day 75.5 ± 8.2 after postpartum/post-induced luteolysis. The same day, a single dose of 10 µg/kg *im* of EB[4] was applied. Twenty four hours later (d+76), 0.8 µg/kg *im* DA[5] every 24 hours

[1] http://www.ema.europa.eu/docs/en_GB/document_library/Maximum_Residue_Limits_-_Report/2009/11/WC500013629.pdf (fecha de consulta: 1 de abril de 2014)

[2] BioRelease deslorelin ®, BET Pharm, Lexington, KY, USA

[3] Estrumate® Vet Friesoythe Pharma GmbH, Germany

[4] Estrozoo® Laboratories Zoo Bogota-Colombia SAS

[5] Sucromate®, 1044 E. Thorn BioScience LLC Louisville, KY 40204, USA

was administered until the preovulatory LH surge (sP4 between 1.5 and 2.5 ng/mL) (figure 1). The first day of proestrus was determined when bitches presented vulvar edema or bloody vulvar discharge or a VC profile > 10% of squamous surface cells. After initiating the administration of DA, sP4 was measured when the VC profile presented > 40% superficial cells and was repeated every two days until a_value between 1.5 and 2.5 ng/mL was reached. Ovulation was confirmed by measuring sP4 levels when the last insemination was performed to the bitches.

VAGINAL CYTOLOGY AND SERUM PROGESTERONE MEASUREMENTS

Samples were taken indirectly to monitor serum estrogen levels before, during and after treatment with EB and DA, starting on day 75±9 of anoestrus, just before the administration of EB, and repeated every two days until the start of cytological dioestrus. Samples were obtained by introducing a cotton-tipped swab moistened with physiological saline (NaCl 0.9%) into the vagina and gently rotating it against the floor and lateral walls of the vagina. The swab was gently rolled over a glass slide, and the smears were air-dried and stained with a drop of methylene blue. Stained slides were examined at 400X to count 200 epithelial cells per slide and classified as parabasal, intermediate, and superficial cells according to Post (1985).

To quantify sP4 levels, cephalic blood samples were collected and serum obtained. All samples were processed within three hours using the chemiluminescence enzyme immunoassay[6] method. The results were always obtained within six hours after collecting the samples.

ARTIFICIAL INSEMINATION

All bitches were artificially inseminated with fresh semen three and five days after treatment when progesterone levels were between 1.5 and 2.5 ng/mL. The same person performed all inseminations. Semen from any of three healthy dogs with proven reproductive performance and fertility by recent results of semen analysis was used.

STATISTICAL ANALYSIS

Descriptive statistics (mean, standard deviation, median and range) were calculated from the start of the treatment with DA to the onset of the proestrus interval, the start of treatment to the day of the LH surge-expected inteval, and the inter-estrus interval (from the preceding estrus to the induced estrus). We calculated the proportion of proestrus-induced bitches, of ovulating bitches, and of bitches that became pregnant. The Wilcoxon Rank-Sum nonparametric statistical test was applied. Data were analysed using statistical software[7].

[6] Cobas e411®, Roche Diagnostics

[7] SPSS® v18.0.

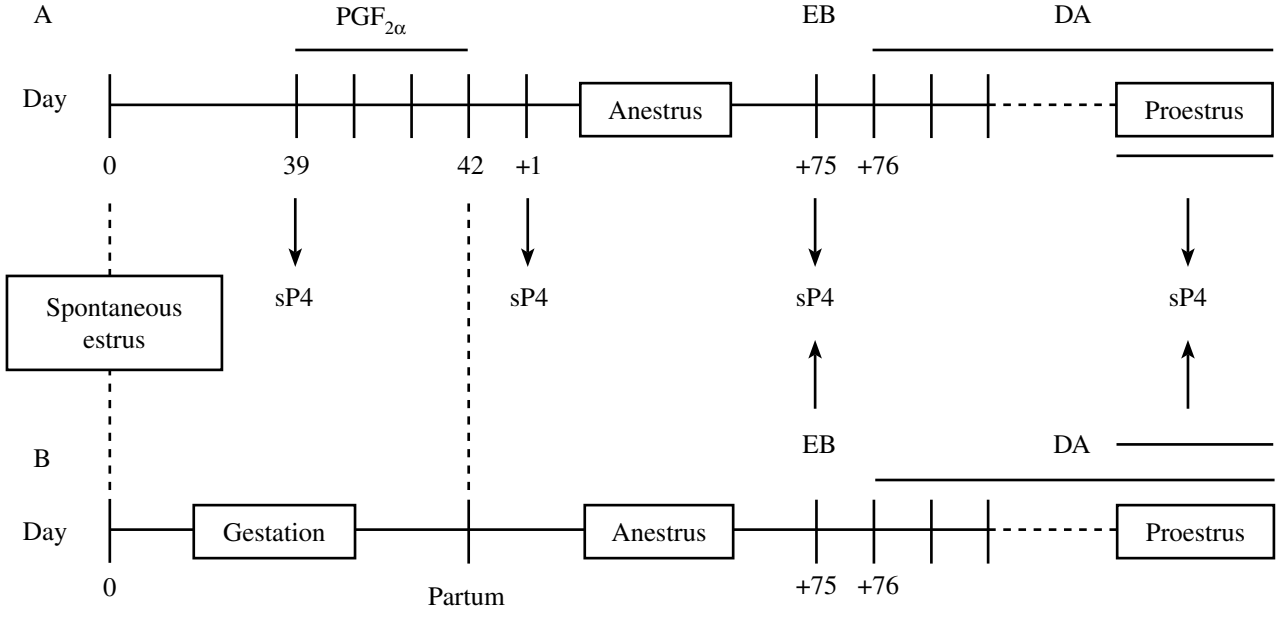

Figure 1. Hormonal treatment protocol and serum progesterone measurements. A: dioestrus bitches, B: postpartum anoestrus bitches, $PGF_{2\alpha}$: prostaglandin, EB: estradiol benzoate, DA: desolerin acetate, sP4: serum progesterone, +: days of start of anoestrus.

Protocolo de tratamiento hormonal y medición de progesterona sérica. A: perras en diestro, B: perras en anestro posparto, $PGF_{2\alpha}$: prostaglandina, EB: benzoato de estradiol, DA: acetato de desolerina, sP4: progesterona sérica, +: días desde el inicio del anestro.

RESULTS AND DISCUSSION

$PGF_{2\alpha}$-induced luteolysis in diestrus bitches was successful on the fourth day of treatment (sP4 < 1 ng/mL +1d) (table 2). One animal had two episodes of diarrhoea after the first $PGF_{2\alpha}$ administration and all dogs presented vomiting and abdominal pain, although these effects were self-limiting and lasted one hour. The luteolysis protocol was effective using a low dose of $PGF_{2\alpha}$, a longer interval between applications and the lowest number of days, as reported by Eilts (2002), in order to reduce undesired effects of the hormone. The bitch´s luteolysis determines the onset of anoestrus and the endometrial histological repair needed for the next proestrus and uterine preparation for a possible pregnancy. Endometrium repair occurs between days 45 and 90 after sP4 levels drop to < 1 ng/mL (Groppetti 2010). We applied $PGF_{2\alpha}$ at mid diestrus to start these events and reduced the interestrous interval by at least 30 d. We can assume that a voluntary waiting period of 75 d was enough to complete uterine repair, since bitch ED2 (excluded due to spontaneous estrus) presented normal proestrus and estrus was inseminated and endometrial and luteal function allowed a normal gestation term.

All treated bitches showed proestrus signs on day 4.5 ± 0.9 (range 3.5-6) after the start of administration of DA. Bitch ED2 presented signs of proestrus (bloody vulvar discharge, vulvar and vaginal edema, VC > 40% of superficial cells) on +75 d, the day when EB administration would begin, so it was excluded from statistical analyses. The other bitches were in anoestrus on +75 d before starting hormonal proestrus induction, as confirmed by the absence of external heat signs, VC results of < 5% of superficial cells and low sP4 (< 0.5 ng/mL). The single and low EB dose was administered to induce the anterior pituitary GnRH receptor and the ovarian LH and FSH receptors (Tani *et al* 1997, Tani *et al* 1999), without observing side effects (Weiss 2003, Sontas *et al* 2009, Schnelle and Barger 2012). The DA protocol starting on day 78 was effective to induce proestrous at 4.5±0.9 d after it had begun in all bitches. No inflammation, swelling, heat and redness were observed at the injection sites of DA in any animal; no side effects were detected. In this study, the pituitary gland was responsive to DA producing increased gonadotropin levels that in turn caused a response in the ovaries. External proestrous signs and VC profile changes suggest that the estrogen level increased by folliculogenesis in response to FSH and LH binding to their receptors probably modulated by EB. Nevertheless, it is not possible to deduce if the EB effect was reached, because the interval from the start of the DA treatment to the beginning of proestrus (4.5±0.9 d), was similar to previous reports in which lutrelyn and desorelin were used without previous BE treatment (Kutzler 2005, Concannon *et al* 2006, Fontaine *et al* 2011, Walter *et al* 2011). It was thus possible to reduce the inter-estrus interval in 71 days, compared to the previous inter-oestrus interval (139 ± 10.4 vs 210.8 ± 14.1 days, respectively) (P = 0.043) (table 2).

All dogs reached sP4 levels between 1.5 and 2.5 ng/mL on day 10.2±2.9 (range 8-15) after the start of DA administration; this was the day when the pre-ovulatory LH surge was expected to occur. Artificial insemination was performed 3 and 5 days later. The progressive sP4 increment, indirectly suggests that luteinization occurs in response to LH amplitude and that frequency pulses rise. On day 10.2±2.8, all bitches reached sP4 levels of 1.5-2.5 ng/mL, in agreement with reports using others GnRH agonists or deslorelin (Concannon *et al* 2006, Lanna *et al* 2010, Fontaine *et al* 2011, Walter *et al* 2011).

sP4 declined to < 1 ng/mL after artificial insemination in 100% of bitches, indicating no corpora lutea formation and ovulation failure. No bitch was determined as pregnant on day 35 by ultrasonography. Bitch ED2 presented a steady and gradual increase in superficial VC cells, sP4 until 2.4 ng/mL, and was inseminated; the bitch became pregnant and gave birth to six viable puppies. Using this protocol

Table 2. Luteolysis and proestrus induction and interestrous interval after hormonal protocol.
Inducción de luteólisis y del proestro e intervalo interestro después del protocolo hormonal.

Animal	sP4 (ng/mL) before $PGF_{2\alpha}$	sP4 (ng/mL) after $PGF_{2\alpha}$	Days after first administration of DA until proestrus	Days after first administration of DA until sP4 between 1.5 to 2.5 ng/mL	Interoestrous interval after hormonal protocol	sP4 levels (ng/mL) after IA
FPP1	6.4	0.5	4	10	144	<1
FPP2	5.7	<0.03	4.5	8	147	<1
FD1	-	-	3.5	10	121	<1
FD2	-	-	4.5	8	143	<1
ED1	21.63	0.8	6	15	140	<1
ED2	5.92	0.07	-	-	-	-
Average	-	-	4.5	10.2	139.0	-
Median	-	-	4.5	10.0	143.0	-
SD	-	-	0.9	2.9	10.4	-

with the dose suggested by Concannon *et al* (2006) (0.6-0.8 µg/kg/d) for lutrelyn, adjusted to the DA short-term release injection protocol, with similar biological potency, all bitches entered the oestrus phase, but they did not ovulate as we expected. We reached this conclusion because in all bitches the sP4 levels declined below 1 ng/mL 3 to 5 d after DA administration was suspended, indicating no corpus luteum formation. Probably this DA dose was not sufficient to induce preovulatory LH surge. With the aim of testing this hypothesis, we administrated a single hCG dose (500 UI *im*)[8] to bitch FD2 (the last experimental bitch) after sP4 levels began to decline from 2.5 to 1.81 ng/mL; 24 and 48 h after hCG administration, sP4 levels reached 5.49 and 9.17 ng/mL respectively, suggesting luteinisation and ovulation due to exogenous hCG administration. This bitch was pregnant as confirmed by ultrasonography and gave birth to eight viable puppies, suggesting that the preovulatory LH surge that was not reached with the DA protocol can be reached by administration of hCG.

In conclusion a single 10 µg/k im EB dose followed by DA administration once a day at a dose of 0.8 mg/kg im for 4.5 days induce proestrus beginning on day 75 of anoestrus, without side effects or changes in the injection site. This protocol is not effective to induce ovulation.

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REFERENCES

Concannon PW, M Temple, A Montanez, L Newton. 2006. Effects of dose and duration of continuous GnRH-agonist treatment on induction of estrus in beagle dogs: Competing and concurrent up-regulation and down-regulation of LH release. *Theriogenology* 66, 1488-1496.

Concannon PW, VD Castracane, M Temple, A Montanez. 2009. Endocrine control of ovarian function in dogs and other carnivores. *Anim Reprod* 6, 172-193.

Concannon PW. 2011. Reproductive cycles of the domestic bitch. *Anim Reprod Sci* 124, 200-210.

Eilts BE. 2002. Pregnancy termination in the bitch and queen. *Clin Tech Small Anim Pract* 17, 116-123.

Fontaine E, F Mir, F Vannier, A Gérardin, M Albouy, C Navarro, A Fontbonne. 2011. Induction of fertile oestrus in the bitch using Deslorelin, a GnRH agonist. *Theriogenology* 76, 1561-1566.

Gobello C. 2007. New GnRH analogs in canine reproduction. *Anim Reprod Sci* 100, 1-13

Groppetti D, A Pecile, S Arrighi, A Di Giancamillo, F Cremonesi. 2010. Endometrial cytology and computerized morphometric analysis of epithelial nuclei: A useful tool for reproductive diagnosis in the bitch. *Theriogenology* 73, 927-941

Kutzler MA. 2005. Induction and synchronization of estrus in dogs. *Theriogenology* 64, 766-775.

Kutzler MA. 2007. Estrus induction and synchronization in canids and felids. *Theriogenology* 68, 354-374.

Lanna LL, AP Marques Jr, RH Douglas. 2010. Effect of deslorelin on the induction of estrus in anestrous bitches. *Arq Bras Med Vet Zootec* 62, 615-621.

Post K. 1985. Canine vaginal cytology during the estrous cycle. *Can Vet J* 26, 101-104.

Root Kustritz MV. 2012. Managing the reproductive cycle in the Bitch. *Vet Clin North Am Small Anim Pract* 42, 423-437.

Schnelle AN, AM Barger. 2012. Neutropenia in dogs and cats: causes and consequences. *Vet Clin North Am Small Anim Pract* 42, 111-122.

Sontas HB, B Dokuzeylu, O Turna, H Ekici. 2009. Estrogen-induced myelotoxicity in dogs: A review. *Can Vet J* 50, 1054-1058.

Stornelli MC, MC García Mitacek, F Giménez, MC Bonaura, I Videla Dorna, RL de la Sota, MA Stornelli. 2012. Pharmacokinetics of eCG and induction of fertile estrus in bitches using eCG followed by hCG. *Theriogenology* 78, 1056-1064.

Tani H, T Inaba, S Matsuyama, Y Takamori, R Torii, H Takano, H Tamada, T Sawada. 1997. Enhancement of estrogen receptor gene expression in the mediobasal hypothalamus during anestrus in the beagle bitch. *Neurosci Lett* 227, 149-152.

Tani H, T Inaba, M Nonami, S Matsuyama, Y Takamori, R Tori, H Tamada, N Kawate, T Sawada. 1999. Increased LH pulse frequency and estrogen secretion associated with termination of anestrus followed by enhancement of uterine estrogen receptor gene expression in the beagle bitch. *Theriogenology* 52, 593-607.

Walter B, C Otzdorff, N Brugger, J Braun. 2011. Estrus induction in Beagle bitches with the GnRH-agonist implant containing 4.7 mg Deslorelin. *Theriogenology* 75, 1125-1129.

Weiss DJ. 2003. New insights into the physiology and treatment of acquired myelodysplastic syndromes and aplastic pancytopenia. *Vet Clin North Am Small Anim Pract* 33, 1317-1334.

Wiebe VJ, JP Howard. 2009. Pharmacologic advances in canine and feline reproduction. *Top Companion Anim Med* 24, 71-99.

[8] Chorulón®, Intervet international™, Boxmeer-Holanda