

Effect of deslorelin on the induction of estrus in anestrous bitches

[Efeito da deslorelina na indução de estro em cadelas no anestro]

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ABSTRACT

The efficacy of one or multiple doses of an injectable formulation of deslorelin (a GnRH agonist) was evaluated to induce estrus in anestrous bitches. Thirteen animals composed three groups: group 1 (n=5, single IM injection of 2mg deslorelin), group 2 (n=5, four IM injections of 2mg deslorelin in alternate days), and control group (n=3, four IM saline injections in alternate days). Daily clinical evaluations, sexual behavior, vaginal cytology, plasma progesterone concentration, ovariectomy and macroscopic evaluation of the uterus and ovaries were done. In group 1, none of the bitches showed signs of estrus, while two developed clinical signs and vaginal cytology of proestrus. In group 2, all animals presented proestrus, four presented estrus, and three ovulated; resulting in a functional corpus luteum and high progesterone concentration until day 25 of diestrus, when ovariectomy was performed. The duration of the stages of deslorelin induced cycles and the progesterone profile were similar to those described in the literature, and no side effects were observed. In conclusion, injectable formulation of deslorelin in multiple injections was effective to induce fertile estrus in anestrous bitches.

Keywords: bitch, deslorelin, induction of estrus

RESUMO

Avaliou-se a eficácia de uma formulação injetável de deslorelina (agonista do GnRH) na indução de estro em cadelas no anestro e seus efeitos no aparelho genital, com uma ou múltiplas aplicações. Treze animais compuseram os grupos: grupo 1 (n=5, 2mg de deslorelina via IM), grupo 2 (n=5, quatro injeções IM de 2mg de deslorelina em dias alternados) e grupo-controle (n=3, quatro injeções IM de solução salina em dias alternados). Foram realizadas avaliações clínicas diárias do comportamento sexual, da citologia vaginal, da dosagem de progesterona plasmática, da ovariectomia e avaliação macroscópica do útero e dos ovários. No grupo 1, as cadelas não exibiram sinais de estro, mas duas apresentaram sinais clínicos e citologia vaginal compatíveis com proestro. No grupo 2, todas apresentaram sinais de proestro, quatro de estro e três ovularam, resultando em corpos lúteos funcionais e concentração elevada de progesterona plasmática até o 25º dia de diestro, quando foi realizada a ovariectomia. Os sinais clínicos, a duração das fases e a curva de progesterona dos ciclos induzidos foram semelhantes aos descritos na literatura, e não foi observado efeito colateral do tratamento. Concluiu-se que a formulação injetável de deslorelina com múltiplas aplicações foi eficaz na indução de estro em cadelas em anestro.

Palavras-chave: cadela, deslorelina, indução de estro

INTRODUCTION

The control of the estrous cycle in bitches, mainly the induction and synchronization of

estrus and ovulation, is fundamental for the application of assisted reproduction techniques. The development of an efficient and predictive method of estrus induction is an important step in clinical approach of infertility in the bitch, and

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should be useful for commercial breeding, resulting in a greater frequency and larger number of births per year. However, the lack of effective protocols of induction and synchronization of ovulation is still an obstacle for the use of reproductive biotechnology in dogs (Concannon, 2005; Kutzler, 2005).

Among the drugs currently available, gonadotrophin-releasing hormone (GnRH) agonists has shown potential for inducing a fertile estrus in bitches (Kutzler, 2007). The deslorelin, a synthetic GnRH agonist, presents potency and stability many times larger than the native hormone (Concannon, 2005), stimulating the secretion of follicle stimulating hormone (FSH) and luteinizing hormone (LH), promoting ovulation (Dyer and Robinson, 1989). Recently, an injectable short-action formulation was developed, tested firstly in mares (Berezowski et al., 2004; Stitch et al., 2004), which raised interest in the study of estrus induction in bitches (Kutzler, 2005).

Based on previous reports and in the potential of GnRH agonists in the induction of estrus in bitches, the present study evaluated the effect of an injectable intramuscular formulation of deslorelin in anestrus bitches, testing one or multiple applications in the induction of estrus and ovulation, and its effects on the reproductive system.

MATERIAL AND METHODS

Thirteen adult mongrel bitches, weighing from 10 to 15kg, aging from about two to seven-year-old, clinically healthy were used. They were brought from the Serviço de Controle de Zoonoses at the Secretaria Municipal de Saúde in Belo Horizonte-MG. All animals were initially characterized in anestrus by vaginal cytology, with predominance of parabasal and intermediate cells, and rare neutrophils, and by the concentration of plasmatic progesterone below 0.5ng/mL, besides the lack of clinical signs of proestrus. A male dog was used in the beginning of the experiment until the cytological characterization of diestrus to evaluate the sexual behavior of the females. During the experiment, the animals were kept in individual cages, fed with balanced commercial food and water *ad libitum*.

The bitches were randomly distributed in three groups: group 1 (n=5, single IM injection of 2mg deslorelin - BET Labs Inc. - Lexington, USA), group 2 (n=5, four IM injections of 2mg deslorelin in alternate days); and control group (n=3, four IM saline injections in alternate days). The day 0 for each animal was defined as the day of the first injection, always in the semitendinous or semimembranous muscle, with the application site being daily evaluated for the presence of edema, pain, or other alterations.

Daily, all bitches were evaluated for the presence of edema and vulvar discharge, as well as for the general clinical condition. The sexual behavior was evaluated by the attraction and receptivity to the male daily exposed to each bitch for five minutes, until the cytological characterization of diestrus.

For the cytological exam, vaginal smears were daily obtained, from the day previous to the beginning of the treatment until obtaining three consecutive smears suggesting diestrus. Following this, every three days until the end of the experiment. The smears were stained using the hematoxilin-Shorr method and evaluated by light microscopy using a 200x or 400x for the proportion of parabasal, intermediate, and superficial cells, as well as for the presence of blood red cells and neutrophils.

Blood samples were daily collected, always in the morning, from the day previous to the beginning of the treatment until diestrus, and following this, every three days until the end of the experiment. The samples were collected by the jugular vein puncture, in 10mL heparinized vacutainer tubes, homogenized, and centrifuged at 700 g for 5min, and the plasma was kept at -20°C until radioimmunoassay which was done with a commercial kit (Progesterone kit coat-a-count - DPC MedLab - São Paulo, Brazil).

Ovaryhysterectomy was done on the 25th day of diestrus post treatment in the bitches which responded to it and at 45 days in the remaining, using the technique and procedures described by Stone (2002). The uterus and ovaries were separated, weighed, and measured for the diameter and length, and the ovaries were inspected regarding the presence of follicles and corpus luteum. Complete sections of 5mm thickness of the average portion of uterine horns

and fragments of the ovaries were collected and fixated in 10% formaldehyde for future histological evaluation.

The data were evaluated by descriptive statistics and by the Student's *t* test at a 5% significance level. Fisher's exact test was used to compare the treatment efficacy between the groups. The experimental protocol was approved by the Comitê de Ética em Experimentação Animal (CETEA/UFMG – protocol nº106/2007).

RESULTS AND DISCUSSION

In group 1, none of the bitches (0/5) presented signs of estrus; however, two presented bloody vulvar discharge and vulvar edema three to six days after the application of deslorelin, which persisted for three to four days, compatible with proestrus signs. Three of the bitches (3/5) attracted the male when challenged, without acceptance of courtship or mating.

In the vaginal cytology, all bitches in group 1 (5/5) presented an increase in the proportion of superficial cells, reaching a high average of $44 \pm 15.2\%$. Both bitches which showed clinical signs of proestrus had over 50% of superficial cells prior to a rapid decline, followed by a predominance of parabasal and neutrophil cells, compatible with diestrus or anestrus. The day of the vaginal smear with less than 20% of the superficial cells was the same as the day of the disappearance of the vaginal discharge and edema in the vulva.

The clinical, cytological, and behavioral signs of proestrus presented by two bitches in group 1 apparently are due to individual response, probably due to the luteinization of some follicles, with and initial synthesis of

progesterone and release of gonadotrophins, although in concentrations not sufficient to induce estrus and ovulation.

Four bitches of group 2, treated with multiple deslorelin injections, showed characteristic signs of proestrus and estrus, including a bloody vulvar discharge, vulvar edema, acceptance of the male, and over 80% of superficial cells in the vaginal smear, differing from group 1 by Fisher's exact test ($P < 0.05$). The signs of proestrus were observed four to six days after the beginning of the treatment (mean 5.0 ± 0.8 days). The mean duration of the proestrus was 8.3 ± 1.0 days. The transition of the proestrus to estrus was defined as the first day in which over 80% of superficial cells were found in the vaginal cytology, and vulvar edema, with or without the vaginal discharge, besides the acceptance or not of the male. The estrus duration was 6.3 ± 0.3 days, while the male acceptance varied from two to six days (mean 3.0 ± 2.2 days).

In the bitches which responded to serial injections of deslorelin, the interval between the beginning of the treatment and the appearance of estrus varied from 12 to 15 days (mean 13.3 ± 1.5 days). The abrupt change of vaginal smear for a typical diestrus occurred 19.3 ± 1.5 days after the first application of deslorelin. One animal of group 2 presented bloody vaginal discharge, vulvar edema, and attraction to the male; however, only reached 65% of the superficial cells, percentage that showed a rapid fall. The appearance of proestrus occurred eight days after the beginning of the treatment and lasted only four days, when the clinical signs ended. Table 1 illustrates the responses obtained in both groups and Table 2 shows the duration of the physiological events in the animals which responded to the treatment.

Table 1. Reproductive responses in bitches treated with one 2mg deslorelin dose (group 1), with multiple 2mg doses (group 2), and saline (control group)

Response	Group 1 n=5 (%)	Group 2 n=5 (%)	Control group n=3 (%)
Proestrus	2 (40)	5 (100)	1 (33)
Estrus	0 (0)	4 (80)	0 (0)
Ovulation	0 (0)	3 (60)	0 (0)
Luteal phase	0 (0)	3 (60)	0 (0)

Table 2. Appearance of reproductive events, in days, from the beginning of the treatment in bitches treated with one 2mg deslorelin dose (group 1), and with multiple 2mg doses (group 2)

Event	Group 1	Group 2
Proestrus	4.5±2.1 (n=2)	5.6±1.5(n=5)
Estrus	-	13.3±1.5(n=4)
Ovulation	-	16±2(n=3)
Diestrus	-	19.3±1.5(n=3)

In the control group, one of the bitches kept with the treated ones presented proestrus signs 35 days after the beginning of the experiment, apparently without any bioestimulation, or “dormitory effect”, from other animals. The other two showed clinical signs, vaginal cytology, and sexual behavior which were compatible with anestrus during the period of the experiment.

One bitch in group 1 and two in group 2 showed pain and light edema on the site of deslorelin application, with one of them presenting discreet claudication for two days, with no alteration in disposition or appetite. No local or systemic treatment was used. No other collateral effect was observed, being the liquid formulation of deslorelin considered safe, in compliance with studies that evaluated the safety of GnRH agonists (Tarlantzis e Bili, 2004).

In all animals of group 1, it was found a discreet elevation of the plasmatic progesterone in the first days after the application of deslorelin. In three animals of this group which did not present clinical signs of proestrus, and in the other two that showed proestrus clinical signs, the progesterone values remained below 1ng/mL following the treatment and during all days of the experiment, which suggests that one dose of deslorelin was inefficient to induce estrus in anestrous bitches. These results are similar to those previously reported, that showed the need to stimulate the bitches with GnRH for a period of seven to fifteen days in order to obtain a consistent ovarian response (Vanderlip et al., 1987; Concannon et al., 1989; Concannon et al., 1997; Kutzler, 2007).

The mean concentration of progesterone in group 1, from days 3 to 6 after the treatment, was higher in the animals that showed proestrus than in the others ($P<0.05$). After day 15, all animals

of the group presented progesterone concentration equal or below 0.1ng/mL, which remained in this value most of the time, with discreet oscillation in this period.

In group 2, four bitches (4/5) presented progesterone values above 1ng/mL, two to three days before the first clinical signs of estrus, differing from the observed in group 1 ($P<0.05$). Three bitches ovulated (3/5), considering the indicated by progesterone values above 4ng/mL and later increase in the values until diestrus. These animals maintained the progesterone concentration close to or above 15ng/mL until the 25th day of diestrus, until they were submitted to ovariectomy and found corpora lutea, differing from those which did not present estrus ($P<0.05$). The progesterone peak in bitches in this group was 23.8±2.1ng/mL, similar to the findings by Cain et al. (1988); however, lower than that found by Concannon et al. (1975). The ovulation day was estimated as the first day in which the plasmatic progesterone surpassed the value of 4ng/mL, resulting that in three bitches the ovulation occurred on the second or third day of the induced estrus, similar to the observed by Phemister et al. (1973) and De Gier et al. (2006), 16±2 days after the first of fourth injection of deslorelin. One animal presented clinical signs, vaginal cytology, and sexual behavior of estrus, similar to the other responsive bitches; however, it did not ovulate. The progesterone concentration increased up to 2.1ng/mL, 12 days after the beginning of the treatment, similar to the observed in the three animals which ovulated ($P>0.05$), when it began to decrease, returning to basal values after six days.

One bitch of group 2 presented signs of proestrus; however, did not show estrus, with the progesterone concentration near 1ng/mL and, presumably, did not have the LH peak, which resulted in the end of proestrus. The mean progesterone concentration in this bitch was not different from those in group 1 which presented signs of proestrus; however, it was significantly different from those bitches which presented estrus after the treatment ($P<0.05$). It can be speculated that it reached basal values of progesterone recently, being in a very initial stage of anestrus and presenting inadequate LH stock in the hypophysis (Fernandes et al., 1987), insufficient to complete the follicular development and ovulation. Another considered

hypothesis would be low sensibility and response of the hypophysis at the time of stimulation by the GnRH agonist (Concannon et al., 1997), as occurs during anestrus of normal bitches (Okkens and Kooistra, 2006).

It is not possible to clarify the reason for the ovulation failure in one of the bitches in group 2, although it had showed similar estrus signs as the bitches which responded with ovulatory estrus. Similar situation was described in bitches treated with LH (Concannon et al., 1989; Verstegen et al., 1997), GnRH (Cain et al., 1989; Concannon et al., 1997), or GnRH agonist (Volkman et al., 2006). The initial progesterone increase and its subsequent decrease during estrus suggest that the LH surge was insufficient to promote ovulation of the follicles initially recruited. This phenomenon, named as "false estrus" by some authors, has been registered in spontaneous ovulation failures and does not seem to be related to the circulating estrogen, given that the vaginal cytology and sexual behavior were not altered (Concannon et al., 1997).

Of the animals in group 2, three presented proestrus, estrus, and ovulation and the development of corpora lutea similar to the described in spontaneous estrous cycles (Concannon et al., 1975; Feldman and Nelson, 2004) and cycles induced by GnRH agonists (Cain et al., 1989; Concannon et al., 1997). The response found in the present treatment (60%) is similar or even higher than results found in previous reports (Concannon et al., 1989; Concannon et al., 1997; Verstegen et al., 1997), considering the lack of knowledge of the anestrus stage or previous fertility history of the used bitches (Volkman et al., 2006). Tests with one or multiple applications of deslorelin had not been efficient in the induction of canine estrus (Kutzler, 2007), although preliminary results suggested the potential of the use of only one dose of the drug (Kutzler, 2005). Kutzler (2007) tested, without success, one, two, and three IM applications of 1.5mg deslorelin, in alternate days. It can be speculated that the response found in the present study is due to the longer treatment period and to the higher deslorelin dose used in the group 2, resulting in satisfactory stimulation of the hypophysis and induction of estrus and ovulation, differing the results found by Kutzler (2007).

The three bitches in the control group maintained progesterone concentration close to or below 0.1ng/mL until day 35 of the experiment, differing from the observed in bitches in groups 1 and 2 ($P<0,05$). From that time on, two bitches continued with basal progesterone values, while one of them presented a slow and progressive increase until the end of the experiment, reaching 1.3ng/mL on day 45 (the last dosage included in the experimental data), being the progesterone curve, the clinical and cytological signs compatible with the beginning of an estrous cycle.

The ovulation in three animals of group 2 was confirmed after ovariectomy by the presence of corpora lutea, with a total of 14 of them, with an average of 4.7 ± 1.2 per animal and 2.3 ± 1.0 per ovary, similar to the findings in estrus induced with deslorelin implants (Volkman et al., 2006). The weight of the ovaries was significantly higher in the bitches in Group 2 that ovulated, being $2.8\pm 0.7g$ versus $1.2\pm 0.5g$ in the others, due to the contribution of the corpora lutea (Ström Holst et al., 2001). None of the bitches had corpus luteum and follicles simultaneously when examined after the ovariectomy. No difference was found in the size of the uterus among groups ($P>0.05$). The average diameter of the uterine horns was $7.0\pm 0.8mm$, $7.4\pm 0.6mm$, and $6.9\pm 0.7mm$, in groups 1, 2, and control, respectively. The average length of the uterine horns was $9.0\pm 1.3cm$, $9.3\pm 1.6cm$, and $8.7\pm 1.5cm$, respectively in groups 1, 2, and control. In none of the animals there were macroscopic signs of pathological lesions in the genitalia.

The anestrus stage of the bitches in the present study was not characterized from the previous estrous cycle, which was not known. Due to this, it is not possible to be sure if the effect of the deslorelin injection was only on the induction of estrus or either on the reduction of interestrus intervals, as previously suggested for the effect of the anestrus stage on the treatment response (Van Haafte et al., 1994; Verstegen et al., 1999). However, in the present study, there is evidence that the deslorelin was able to induce proestrus, estrus and ovulation, when administered in a serial manner, in alternate days, with intramuscular application, in the used dose.

CONCLUSIONS

The formulation of 2mg deslorelin proved to be effective in the induction of estrus in anestrus bitches, when administered in alternate days, via intramuscular and in a total of four applications. The estrus induced by deslorelin, in its clinical characteristics, was similar to the spontaneous estrus described in the literature.

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