American Journal of Animal and Veterinary Sciences 3 (1): 43-46, 2008
ISSN 1557-4555

Noninvasive Measurement of Fecal Progesterone Concentration in Toy Poodles by Time Resolved Fluoroimmunoassay (TR-FIA)

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Abstract: Progesterone is an important reproductive hormone and measurement of its level by repeated blood samplings is beneficial to monitoring of estrus cycle. However, since toy poodles have a small body size and thin-walled blood vessels, repeated blood samplings cause stress and affect their preparation for mating or artificial insemination (AI). Therefore, a noninvasive method for monitoring progesterone concentration should be developed. Here, we show that time-resolved fluoroimmunoassay (TF-RIA) is a useful noninvasive method for determining the progesterone concentration in serum and fecal samples obtained from toy poodles. Present results demonstrate that progesterone concentrations in the fecal correlated with the serum collected in same time and the sequential changes in progesterone concentrations in the feces are paralleled in the serum. Therefore, this technique may be suitable for monitoring the estrus cycle in toy poodles.

Key words: Fecal, Progesterone concentration, Time-resolved fluoroimmunoassay, Toy poodle

INTRODUCTION

Progesterone is a hormone that controls many aspects of reproduction in female mammals, such as preparation of the endometrium for embryo implantation, maintenance of pregnancy, gonadotropin secretion and sexual behavior [1].

In domestic dogs, progesterone levels have been monitored through repeated blood samplings to determine the various stages of the estrus cycle, pregnancy diagnosis and to determine the optimal mating or artificial insemination (AI) time [2-4]. However, monitoring the progesterone levels in small-breed dogs such as the toy poodle by blood sampling has limitations. These include small body size and thin-walled blood vessels of the toy poodle; moreover, the stress of repeated blood samplings affects the dog's preparation for mating or AI. Therefore, a less stressful and easily repeatable sampling method such as fecal progesterone analysis would be beneficial for the small breed dogs such as toy poodles.

Radioimmunoassay (RIA) and enzyme immunoassay (EIA) are the most commonly used techniques for fecal progesterone analysis. RIA procedures must be performed in a special area controlled by the highly -sensitive regulation. Although EIA is less sensitive than RIA, it dose not require a special facility. Therefore, it provides an alternate, simple and highly sensitive procedure for fecal progesterone analysis in toy poodles that can be performed in a conventional area without the use of hazardous material.

For the time-resolved fluoroimmunoassay (TR-FIA), a lanthanide labeled tracer is used. TR-FIA is based on time-resolved fluorometry and not radioactivity counting [5]. This assay has been applied for serum progesterone analysis in humans, bovines and Iberian red deer and reliable results have been obtained [5-7].

The objective of this study was to determine the fecal progesterone concentration by TR-FIA, to determine if the fecal progesterone concentration was correlated with serum progesterone concentration.

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MATERIALS AND METHODS

Animals: Adult female toy poodles of various ages and parity (n = 20) were used in this study. They were housed individually under the condition of 28°C at Japan Animal Specialty College, Sendai, Japan. The animals were fed commercial concentrate feed and water and maintained without any restraint except when blood samples were collected.

Blood and fecal sampling: Blood samples (1 mL) were collected via jugular venipuncture. After clotting, the serum was separated by centrifugation at 3000 g for 5 min. Fecal samples (approximately 10 g) were collected after excretion of fecal within 30 min and placed in freezing bags.

Extraction of fecal: A 1 g portion of each fecal sample was placed in a 15 mL polypropylene tube and 5 mL of 100% of methanol was added. The tubes were vortexed for 5 min and shaken for 10 min. The samples were then centrifuged for 15 min at 3000 rpm and the supernatants were decanted into polystyrene tubes, methanol was removed completely using a centrifugal vacuum concentrator and added 500 μL of the TR-FIA assay buffer was added.

Progesterone assay: Serum and fecal progesterone concentrations were measured using time-resolved fluoroimmunoassay, DELFIA^{TM} progesterone kit (PerkinElmer Life Sciences, Wallac Oy, Turku, Finland) the according to the manufacturer's instructions. Briefly, the fecal extract, serum sample, or reference standard of progesterone and the antibody and Europium-labeled tracer were dispensed into an assay plate (96 well, precoated with secondary antibody). The plate was shaken slowly for 2 h at room temperature, washed and an enhancement solution was added. Fluorescence was detected using the Wallac Fluorometer with a 5 min enhancement. The progesterone assay for both the fecal and serum samples was performed in duplicate. The recoveries rate of progesterone standard added to feacal samples were 75.8 ± 13.2%. The minimum detectable level of progesterone by TR-FIA was 7.75 pg/well.

RESULTS AND DISCUSSION

The intra and inter variations, expressed as the coefficient of variation (CV), was determined by analyzing 3 samples. The intra-assay CV ranged between 1.1 and 6.3%. The sample with high progesterone concentration had a low CV. On the other hand, the CV for the inter-assay precision was between 5.4 and 13.4%. The correlation between serum and fecal progesterone concentration is shown in Fig. 1. The progesterone concentration per gram of feces was considerably higher than that per milliliter of serum. The linear regression formula was as follows, Y = 4.2 X+131.2 (n = 55, Y = fecal content, ng g^{-1} feces, X = serum concentration, ng mL^{-1}). The correlation (r = 0.51) between the serum and fecal progesterone concentrations was statistically significant ($p < 0.001$).

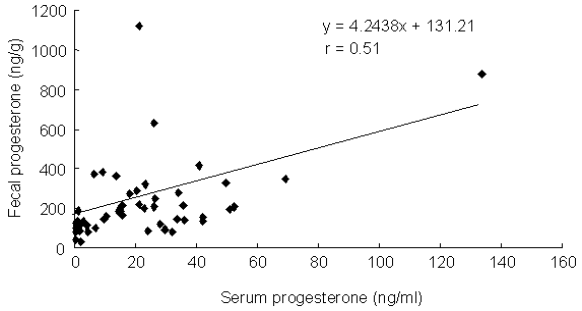

Fig. 1: Correlation of progesterone concentration in the serum and fecal samples at various stages

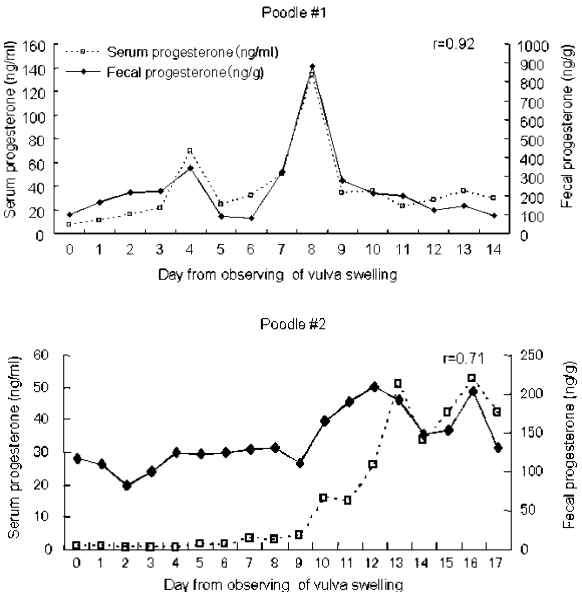

Fig. 2: Changes in the serum and fecal progesterone concentrations during the estrus cycle in 2 canines. Day 0 is the day when swelling of vulva was observed

The results of monitoring the fecal progesterone concentration in 2 poodles during the estrous cycle are shown in Fig. 2. In both poodles #1 and #2, the sequential changes in the progesterone concentrations

in the feces were paralleled in the serum during the estrus cycle. Statistically significantly ($p < 0.001$) strong correlations of $r = 0.92$ and $r = 0.71$ were observed between the serum and fecal progesterone concentrations for poodles #1 and #2, respectively.

All the correlation coefficient were obtained using simple regression analysis (Excel software).

The results of the present study demonstrated that there was a strong correlation between the progesterone concentration of the serum and fecal samples assessed by time-resolved fluoroimmunoassay (TR-FIA) using DELFIA™ progesterone kit at various stages of the estrus cycle for individual toy poodles. This suggested that measuring of the fecal progesterone concentration could be used for noninvasive analysis of estrus monitoring. The present study is the first to report that the fecal progesterone concentration in dogs can be measured by TR-FIA using DELFIA™ progesterone kit.

In the present study, the correlation between the blood and fecal samples ($r = 0.51$) of toy poodles is similarly to that reported previously for domestic dogs by Hay *et al*. and Gudermuth *et al*., using a EIA system [8, 9]. TR-FIA has often been described as a more sensitive and easier method as compared to EIA [10]. Morever, this protocol was simplified by using a commercially available DELFIA™ progesterone kit. Hence, this protocol is believed to be the optimal and easy method for monitoring the estrus cycle.

Determining the actual ovulation time is more difficult in bitches than in other mammals [9, 11]. This is because in bitches, there is no fixed interval between the rise in progesterone levels and ovulation. Since the interval between the rise in progesterone levels and ovulation can range from 1.5 to 4.5 days, examining the longitudinal changes in serum progesterone patterns by repeated blood sampling is the most common methods for estimating the time of ovulation and the optimal time for insemination in dogs [11]. This timing is particularly important when planning AI with frozen/thawed canine semen because of the short lifespan of cryopreserved semen as compared to fleshly collected canine semen [11-13]. Recently, we developed a new artificial vagina (AV) for the collection of semen from toy poodles and a method for freezing with a trehalose-egg yolk extender [14]. However, because blood sampling is difficultly for small breed dogs such as the toy poodle that has thin-walled blood vessels and since the measurement of progesterone concentration by repeated blood samplings stresses canines, noninvasive measurement of progesterone concentration is suitable for preparing the dogs for AI with cryopreserved semen [9].

The previous protocol used for assaying fecal progesterone concentration was lengthy; at least 2 days were required for fecal extraction and steroid analysis [9]. Therefore, miss timing of the appropriate time for AI with cryopreserved sperm was possible. However, in the present study, extraction and the progesterone assay of flesh fecal samples could be performed in a short time (approximately 10 hr). Moreover, in the present study, we showed that the fecal progesterone concentration dramatically increasesed (> 160 ng g^{-1}) from the ovulation time, which is the period when the progesterone concentration in the serum is more than 5 ng mL^{-1} [11]. Therefore, for toy poodles, TR-FIA could be provide a better prediction of ovulation time and the efficient obtaining of pups if the present technique is combined with the used of AV for collecting highly motile sperm from and frozen/thawed sperm.

In conclusion, the present study demonstrated that there is a high correlation between the serum and fecal progesterone concentration by monitoring these levels using TR-FIA; this non-invasive method could be utilized to determine of the optimal time for AI in toy poodles.

ACKNOWLEDGEMENT

This study was partly supported by a grant from the Japan Animal Specialty College (to Eimei Sato).

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