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Application of Chemiluminescence Enzyme Immunoassay Method to Collect *in vivo* Matured Oocyte in Dog Cloning

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Abstract : Accurate determination of *in vivo* oocyte maturation is particularly critical for dog cloning compared to other assisted reproductive technologies because oocytes in metaphase II stage have to be recovered in order to undergo somatic cell nuclear transfer right after its recovery. The aim of present study was to evaluate the reliability and to set a reference range of a chemiluminescence enzyme immunoassay (CLEIA) compared to radioimmunoassay (RIA) method to retrieve *in vivo* matured oocytes. Serum progesterone concentration during proestrus and estrus was analyzed by RIA and CLEIA to determine ovulation day (Day 0). On Day 3, *in vivo* oocytes were recovered surgically and evaluated microscopically maturation status after staining nucleus with bisbenzimidazole dye. Mean progesterone concentration by CLEIA (7.64 ± 0.06 ng/ml) was significantly higher than by RIA (6.46 ± 0.04 ng/ml, $P < 0.0001$). It was not different between CLEIA (10.01 ± 0.34 ng/ml) and RIA values (7.91 ± 0.14 ng/ml, $P < 0.05$) on Day 0, but significantly higher CLEIA level on Day -1 and Day 1 (6.41 ± 0.15 and 14.25 ± 0.44 ng/ml) was assessed compared to RIA (4.95 ± 0.10 and 11.29 ± 0.34 ng/ml). However, with both methods, progesterone level was significantly increased from Day -1 to Day 2. To determine oocyte maturation with CLEIA method, a wider and higher reference range has to be considered.

Key words : chemiluminescence enzyme immunoassay, *in vivo* matured oocyte, progesterone, dog.

Introduction

Oocytes in metaphase II (MII) stage is one of the key materials in assisted reproductive technologies including artificial insemination and cloning due to high maturation promoting factor concentrations in the MII cytoplasm is essential to lead to somatic cell nuclear envelope breakdown, chromosome condensation, reorganization of the cytoskeleton and changes in cell morphology (14,16,17). Compared to artificial insemination, the more delicate determination of the MII stage is necessary for dog cloning because artificial insemination can be performed even when the *in vivo* oocytes are in immature stage due to the long lifespan of spermatozoa in female reproductive tract (7). However, for dog cloning, oocytes in matured stage have to be retrieved because the varying and poor results of *in vitro* maturation system in this species (usually around 20%; range 0-79.5%) (3,22) compared in farm animal (75-90%) (20,21). Also, while it is possible that twice of intrauterine insemination on the second and third day after estimated ovulation *via* non-surgical approach (23), repeated recovery of *in vivo* oocytes is not possible because more than 93% of oocytes are retrieved from an oocyte donor dog by surgical retrieval (9). Up to now, several studies have been reported a range of progesterone concentration with radioimmunoassay (RIA) method to retrieve *in vivo* oocyte maturation (9-11), but chemiluminescence enzyme immunoassay (CLEIA) method has not yet

been applied in dog cloning. Therefore, the aim of the present study was to evaluate the reliability and to set a reference range of CLEIA compared to RIA method to determine *in vivo* oocyte maturation for dog cloning.

Materials and Methods

Mixed breed female dogs aged between 1 and 5 years and weighing 20 to 35 kg were used in this study. Animal care facilities and procedures followed the standards established by the Committee for Accreditation of Laboratory Animal Care at Seoul National University. The use of animals in this study was according to the Guide for the Care and Use of Laboratory Animals at Seoul National University.

Blood was collected from dogs in proestrus/estrus every day or every other day. Serum progesterone concentration was measured with a DSL-3900 ACTIVE® Progesterone Coated-Tube Radioimmunoassay Kit (Diagnostic Systems Laboratories, Inc., Webster, TX) for the RIA method (9-11), and with a Immulite 1000 (Siemens Healthcare Diagnostics Inc., Flanders, NJ) for the CLEIA method.

Ovulation day (Day 0) was determined when serum progesterone results by RIA reached at 6-10 ng/ml (10). On day 3, *in vivo* matured oocytes were recovered by flushing fallopian tubes surgically as described in a previous study (13). Recovered oocytes were stained with Hoechst 33332 and maturation status was assessed by their perivitelline space (PVS) and polar body (PB) extrusion under a micromanipulator (Nikon-Narishige, Tokyo, Japan). Oocyte without first PB was regarded as immature, and among the others, oocytes

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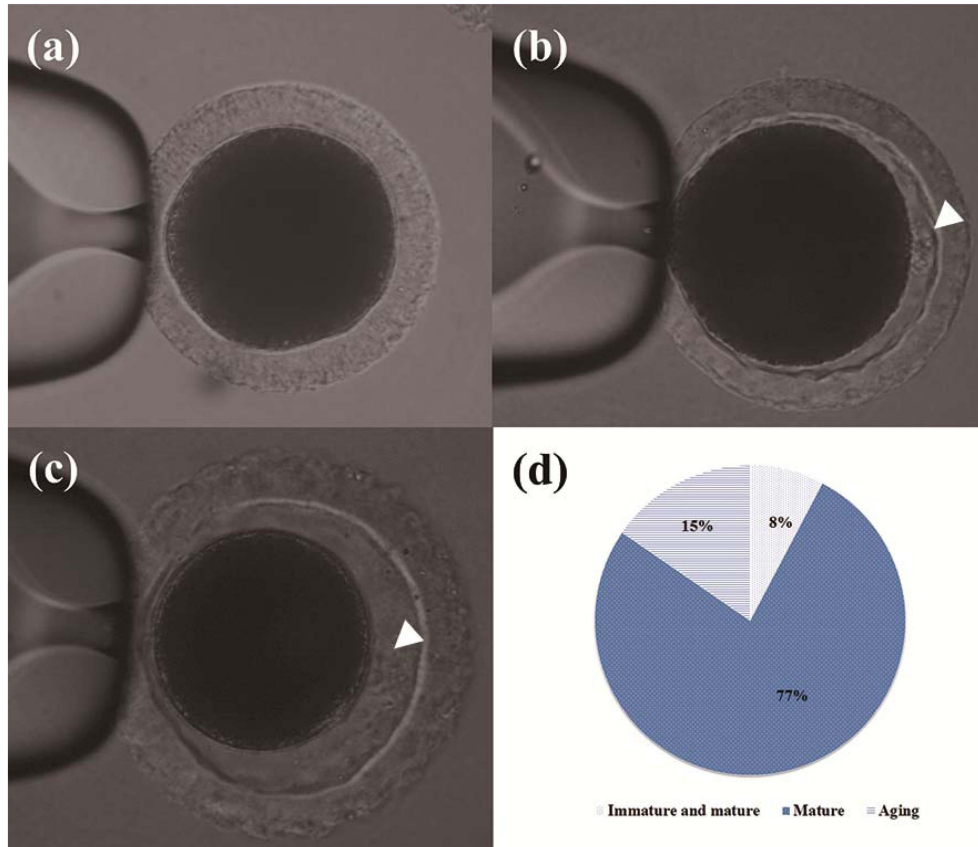
Fig 1. Classification of recovered *in vivo* oocytes. Oocytes were categorized as immature (a), mature (b) and aged (c) depending on the presence of a polar body (white arrow head) and the width of the perivitelline space. (d) Represents the percentage at each stage of the oocytes recovered *in vivo* in this study.

with PVS < 25 μm and > 25 μm were classified as mature and aging, respectively (Fig 1a-c) (5).

In experimental 1, progesterone concentrations measured by RIA and CLEIA methods from 22 bitches were compared during proestrus and estrus. Mean serum progesterone concentrations measured by both methods were compared by a paired t-test, and correlations between the concentrations were analyzed by Bland-Altman analysis and Deming regression. In experiment II, after evaluating and categorizing the *in vivo* recovered oocytes, data from the dogs yielding mature oocytes were compared from Day -2 to Day 1. The Wilcoxon matched pairs test was used to compare the progesterone results on each day and daily increases from Day -2 to Day 1. Data were analyzed using Graph Prism software (GraphPad, San Diego, CA), and the significance level was considered when $P < 0.05$.

Results

A total of 73 blood samples form 22 bitches was collected, and mean progesterone concentration was significantly higher in CLEIA (7.64 ± 0.06 ng/ml) than in RIA (6.46 ± 0.04 ng/ml, paired t-test, $P < 0.0001$) results. However, progesterone levels measured with CLEIA and RIA showed a good correlation by Bland-Altman analysis (bias = 1.18; 95% CI = −3.05 to 5.40), and Deming regression analysis (slope = 0.75; 95% CI = 0.64 to 0.86, intercept = −0.95) (Fig 2). Also, most of the differences between CLEIA and RIA for average ovula-

tory progesterone concentrations (4.0 to 10.0 ng/ml) (7) were within ± 1.96 SD (Fig 2b).

Among the 22 bitches, *in vivo* matured oocytes were recovered from 13 dogs. Matured and aged oocytes were recovered from 10 ("matured" group) and 2 dogs ("aged" group), respectively, and both immatured and matured oocytes were recovered from 1 dog ("immatured" group; Fig 1d). The mean progesterone concentration in the matured group were not significantly different by the assay methods on Day -2 (3.81 ± 0.17 ng/ml by RIA and 4.27 ± 0.13 ng/ml by CLEIA) and Day 0 (7.91 ± 0.14 ng/ml by RIA and 10.01 ± 0.34 ng/ml by CLEIA). But significantly higher progesterone level was measured on Day -1 and Day 1 with CLEIA method (6.41 ± 0.15 and 14.25 ± 0.44 ng/ml) compared with RIA method (4.95 ± 0.10 and 11.29 ± 0.34 ng/ml). Progesterone levels by both RIA and CLEIA methods were similar on Day -2 and Day -1, but it showed significant increase day by day from Day -1 to Day 1 (Fig 3).

Discussion

Although the significance of recovering *in vivo* matured oocytes in dog cloning (6,8), it is still difficult to determine the accurate time of oocyte recovery due to the unique reproductive characteristics in dogs including polyovulation, ovulation at immature stage, and delayed meiotic resumption of ovulated oocytes (7). *In vivo* oocyte maturation has been reported to occur about 54-72 hr (2) or 53-127 hr (19) after
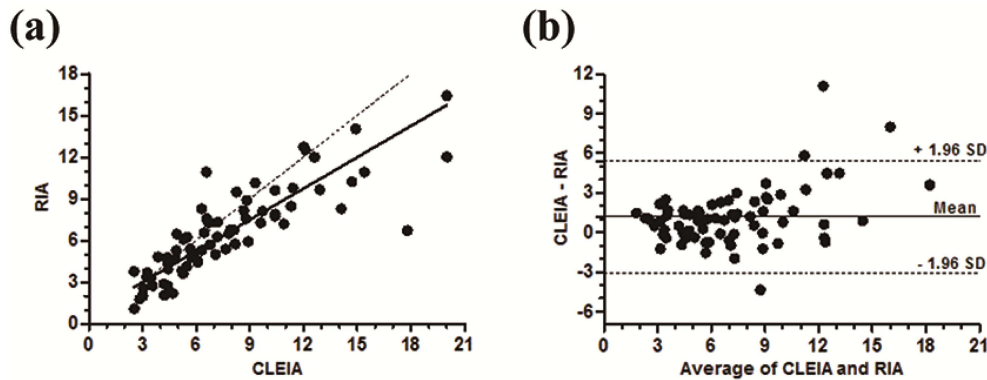
Fig 2. Comparison between RIA and CLEIA methods for progesterone concentration (ng/ml). (a) Deming regression. The dashed line is the line of identity (y = x). (b) Bland-Altman plot. The solid line indicates bias (0.96 ng/ml) and dashed lines indicate the mean ± 1.96 SD (−2.26 and 4.18 ng/ml).
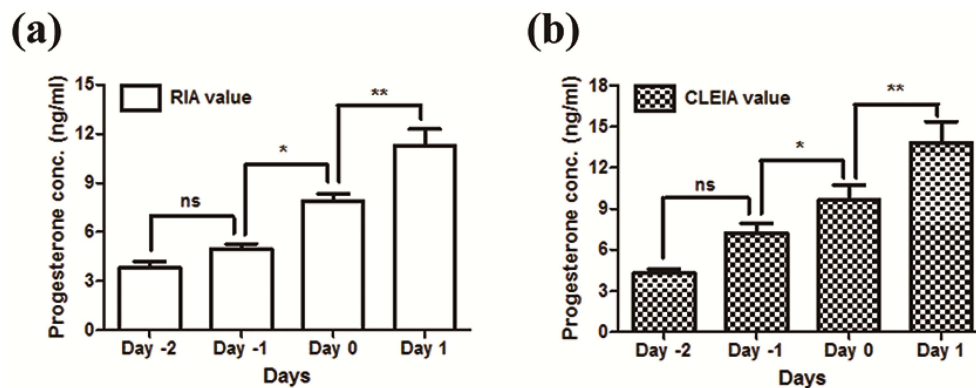
Fig 3. Daily increase of serum progesterone concentration (mean ± SEM, ng/ml) around ovulation. Daily increase pattern was compared according to RIA (a) and CLEIA (b) methods.

ovulation. However, it seems that the duration of *in vivo* oocyte at matured stage in oviducts of an individual dog is relatively short because efficiencies to recover *in vivo* matured oocytes from oocyte donor dog is vary (26.1-72.5%) even with the same RIA method (4,5,18). Therefore, compared with RIA method, reliability of CLEIA method to determine timing of *in vivo* oocyte maturation was examined in the present study.

In line with our results, a higher progesterone level was reported regardless of estrus stage with chemiluminescent assay method compared to RIA method (1). The higher progesterone detection level in CLEIA might be a reflection of higher specificity for the free form of progesterone (1) which is the only biologically active form of progesterone in serum among free, orosomucoid-bound, albumin-bound and globulin-bound forms (12). Thus, for the next step, we focused on setting a reference range with CLEIA compared to RIA method to determine *in vivo* oocyte maturation for dog cloning.

Due to the small numbers of dogs for aged and immature oocytes retrieval in our study, data from only matured group (85% accuracy, Fig 1d) were used to analyze the following study. The dogs donated aged and immature oocytes may represent individual differences of *in vivo* oocyte maturation which occurs about 54-72 hr (2) or 53-127 hr (19). To determine Day 0, besides the average range of ovulatory progesterone concentration, 4.0 to 10.0 ng/ml (7,15), daily increase pattern is also considered as an important indicator due to the wide reference range. For example, while progesterone con-

centration measured by RIA on Day -1 was also within the reference range (7,15), it was not regarded as Day 0 because it did not show a significant increase from Day -2 to Day -1 (Fig 3). Although the higher results were obtained by CLEIA on Day -1 and Day 1, similar tendency was shown that no increase from Day -2 to Day -1 within the reference range (7,15). Also, significant increase from Day -1 to Day 0 and from Day 0 to Day 1 was seen in both RIA and CLEIA (Fig 3). Thus, the significant difference in progesterone levels on Day -1 and Day 1 seems not to influence the daily increase by CLEIA method.

In conclusion, to determine *in vivo* oocyte maturation with CLEIA method, a wider and higher reference range of serum progesterone concentration on Day 0 has to be considered compared with RIA method. Also, daily increase pattern of the concentration can be applied as an indicator with CLEIA similar with RIA method. Further studies are required to analyze a relationship between other reproductive hormone and oocyte maturation for increasing the accuracy of *in vivo* matured oocytes retrieval.

Acknowledgments

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개 복제 시 체내 성숙 난자 회수를 위한 화학발광효소면역분석기법의 적용

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요 약 : 개 복제는 성숙된 중기의 난자를 수술적으로 회수하여 바로 사용해야 하기 때문에 다른 번식 보조술에 비하여 체내 난자 성숙의 정확한 예측이 특히 더 중요하다. 따라서 본 연구는 개에서 체내 성숙 난자 회수 시 방사면역분석기법(RIA)과 비교하여 화학발광효소면역분석기법(CLEIA)의 신뢰성을 평가하고, 참고값을 설정하기 위해 실시되었다. 배란일(Day 0) 결정을 위하여 발정전기와 발정기의 혈청 프로게스테론 농도가 RIA와 CLEIA 방법으로 분석되었다. Day 3에 수술적인 방법으로 체내 난자를 회수하였고, bisbenzimidazole로 핵을 염색한 뒤 성숙을 현미경으로 평가하였다. 평균 호르몬 농도는 CLEIA 값 (7.64 ± 0.06 ng/ml)이 RIA 값 (6.46 ± 0.04 ng/ml, $P < 0.0001$)보다 유의적으로 높았다. Day 0일 때 CLEIA 값 (10.01 ± 0.34 ng/ml)은 RIA 값 (7.91 ± 0.14 ng/ml)과 다르지 않았으나, Day -1과 Day 1일 때는 CLIEA (6.41 ± 0.15 and 14.25 ± 0.44 ng/ml)가 RIA(4.95 ± 0.10 and 11.29 ± 0.34 ng/ml)보다 유의적으로 더 높은 값을 나타내었다. 그러나 두 가지 방법 모두 Day -1에서 Day 2까지 프로게스테론 농도가 유의적으로 점차 증가하였다. 그러므로 CLEIA 방법으로 난자 성숙을 결정하기 위해서는 더 넓은 범위와 높은 참고 값이 고려되어야만 한다.

주요어 : 화학발광효소면역분석기법, 체내 성숙 난자, 프로게스테론, 개