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In vitro effect of recombinant human gonadotropins on meiotic competence of dog oocytes

Kamber DEMİR[1,*], Mithat EVECEN[1], Ramazan ARICI[1], Selin YAĞCIOĞLU[1], Nur ERSOY[1], Nilhan ÇOŞKUN[2], Hatem ATALLA[3], Kemal AK[1], Sema BİRLER[1], Serhat PABUCCUOĞLU[1]

[1]Department of Reproduction and Artificial Insemination, Faculty of Veterinary Medicine, İstanbul University-Cerrahpaşa, Avcılar, İstanbul, Turkey

[2]Translational Medicine Research Center, Animal Research Facility, Koç University, Zeytinburnu, İstanbul, Turkey

[3]Faculty of Veterinary Medicine, An Najah National University, Nablus, Palestine

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Abstract: The reproductive biology of the domestic dog is unique among mammalian species; because of this, in vitro maturation (IVM) rate is still very low compared with other domestic animals in spite of attempts at improvement. The aim of this study was to consider the use of recombinant human gonadotropins as a replacement for pituitary gonadotropins for IVM of dog oocytes. A total of 845 cumulus–oocyte complexes were used in this study. To determine the effects of human recombinant gonadotropins, maturation medium was supplemented with two different concentrations (0.05 or 0.1 IU/mL) of pituitary (pFSH, pLH) and human recombinant (rhFSH and rhLH) gonadotropins. After the IVM period, the maturation rate of the oocytes was investigated under an epifluorescence microscope. Our findings showed no significant difference in maturation rate using either pituitary or human recombinant gonadotropin groups ($P > 0.05$). Applying 1.0 IU human recombinant gonadotropin caused the lowest maturation rate (34.57%; $P < 0.05$). In conclusion, recombinant human gonadotropins could be applied for IVM of dog oocytes. Moreover, 0.05 IU/mL rhFSH and rhLH can be successfully used in place of biologically derived hormones.

Key words: Dog oocyte, in vitro maturation, recombinant gonadotropin hormone

1. Introduction

Dogs and humans are quite similar in physiology, anatomical structure, and responses to drugs and irradiation (1). Moreover, about half of the genetic diseases found in humans are also reported in dogs, which makes them a better model than mice for human hereditary diseases (2). It is known that assisted reproduction techniques such as in vitro maturation (IVM), in vitro fertilization (IVF), and somatic cell nuclear transfer (SCNT) may contribute considerably to protection of endangered species (3,4). In vivo follicle development is arranged by follicle-stimulating hormone (FSH), luteinizing hormone (LH), and inhibin, with coordination of the gonadal–pituitary axis (5). Reproductive physiology of the domestic dog is different from that of most other species; ovulation occurs when the oocyte is immature, and maturation is completed in the oviduct within 48–72 h (6). The proteins produced by LH are responsible for the expansion of the cumulus cells. Expansion of these cells is considered a cytoplasmic maturation indicator in mammals and used for determination of oocyte maturation rates during in vitro fertilization studies. However, cumulus cell expansion is rare and appears to be insignificant for IVM of dog oocytes (7). It is difficult to identify the stimulant or repressive components; the meiotic results of in vitro matured dog oocytes are still unobservable. Furthermore, the success of in vitro technologies is still very limited in dogs (4,8). Previously, our attempts to mimic the in vivo hormone secretion mechanism of dogs showed an in vitro maturation rate lower than 50% (9). The low IVM rates in dogs are the main cause of IVF and subsequent embryonic development failures (4). To our knowledge, only one blastocyst (10) and two morula-stage embryos (10,11) have been achieved using in vitro fertilization of immature dog oocytes. Despite live pups being born from SCNT of dog oocytes, success of in vivo maturation (12), and detection of one pregnancy (13), no offspring have been reported by transferring embryos produced from in vitro matured canid oocytes. Moreover, results of studies using in vivo matured dog oocytes have also been disappointing. Only seven (14) and six (15) healthy puppies were delivered in the two most successful trials until date. Reliable systems for in vitro embryo production have not yet been developed, and new approaches are needed for

* Correspondence: kamberdemir@istanbul.edu.tr

dogs (9,11). The cost of pituitary gonadotropins (FSH and LH) is considered an obstacle for in vitro studies in dogs, with low success rates as well. Therefore, the availability of a less costly hormone source may allow for further research. Recombinant human gonadotropins (rhFSH and rhLH) are considered much cheaper than pituitary hormones (pFSH and pLH). These hormones have been used previously for in vitro studies of sheep (16–18), cats (19), humans, cattle, and mice (20,21), and have produced successful results for IVM of oocytes and development rate of embryos. This study was conducted to investigate for the first time the in vitro maturation of dog oocytes using recombinant human gonadotropins.

2. Materials and methods

Ethical approval for animal studies was granted by the İstanbul University Ethics Committee on Animal Research (2018/111555). Unless otherwise mentioned, all chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA).

2.1. Collection of oocytes

Ovaries were obtained from 20 spayed bitches in the anestrous cycle stage after routine ovariohysterectomy at the Avcılar Municipality Stray Animals Sterilization and Rehabilitation Center. Ovaries were maintained and transported to the laboratory in cold physiological saline at 4 °C until oocyte recovery within 2–3 h. Ovaries were left at room temperature for 20 min for rewarming. After slicing, ovaries were washed using HEPES modified TCM 199 washing medium to gather cumulus–oocyte complexes (COCs). Afterwards, COCs were washed using modified synthetic oviduct fluid medium (mSOF) and selected based on a number of criteria, namely being surrounded by at least four cumulus cell layers, and having a dark-pigmented ooplasm and an intact zona pellucida.

2.2. In vitro maturation

In vitro maturation was performed as elucidated in our previous study with minor modifications (9). In brief, the maturation procedure was performed in mSOF medium (270 mOsm, pH 7.2) supplemented with 4% BSA (fraction V), MEM amino acids solution, nonessential amino acids solution, antibiotics, and epidermal growth factor (EGF; 20 ng/mL). The selected COCs were transferred into petri dishes including 50 µL of maturation medium drops covered with mineral oil. Petri dishes were maintained at 38 °C in a humidified atmosphere with 5% CO_2 for 72 h. Depending on the experiment, maturation medium was supplemented with two different concentrations of pituitary FSH (pFSH), LH (pLH), and human recombinant FSH (rhFSH, Gonal-F, Merck, İstanbul, Turkey) and LH (rhLH, Luveris, Merck, İstanbul, Turkey).

To determine the in vitro effect of rhFSH and rhLH on maturation rates of oocytes and to compare them with pituitary gonadotropins (pFSH and pLH), the study groups were designed as follows:

1. Group: Control (no gonadotropin);
2. Group: 0.5 IU pituitary group (0.05 IU/mL pFSH + 0.05 IU/mL pLH);
3. Group: 1.0 IU pituitary group (0.1 IU/mL pFSH + 0.1 IU/mL pLH);
4. Group: 0.5 IU human recombinant group (0.05 IU/mL rhFSH + 0.05 IU/mL rhLH);
5. Group: 1.0 IU human recombinant group (0.1 IU/mL rhFSH + 0.1 IU/mL rhLH).

2.3. Evaluation of nuclear maturation rates

To remove the cumulus cells after the determined in vitro maturation period, oocytes were placed into hSOF medium containing 0.4% (w/v) hyaluronidase and then vortexed for 2 min. Swelling the chromatins was performed by transferring the oocytes to hypotonic KCl solution (0.7%, w/v) for 5 min. The oocyte maturation rates were examined using an epifluorescence microscope after staining with Hoechst 33342 for 20–30 min.

3. Results

3.1. In vitro maturation rate

Maturation rates of dog oocytes are presented in the Table. The highest IVM (metaphase I + metaphase II) rates were found in the 0.5 and 1.0 IU pituitary gonadotropin groups, and these rates were statistically similar (52.41% and 49.73%, respectively; $P > 0.05$). The 0.5 IU recombinant hormone group resulted in a rate of 43.64% without a statistical difference from the 1.0 IU pituitary group (49.73%). However, IVM was lower than in the 0.5 IU treated group. The lowest maturation rate was observed in the 1.0 IU recombinant gonadotropin group (34.57%; $P < 0.05$). Despite the lack of statistical significance, an appreciable decrease in rate of maturation using 0.5 IU recombinant hormone was detected compared with the control group (38.18%). The highest rate of oocytes remaining in the germinal vesicle (GV) stage was found in the 1.0 IU recombinant gonadotropin group (20.99%; $P < 0.05$). However, there were no statistically significant differences between all groups according to germinal vesicle breakdown (GVBD) and undetermined nuclear material (UDNM) parameters (Figures 1A–1F).

3.2. Statistical analysis

The statistical analysis of the study was carried out using Pearson's chi-square test by SPSS 13.0 for Windows (SPSS Corp., Chicago, IL, USA).

4. Discussion

Mammalian oocytes get their meiotic signals via gap junction communication with cumulus cells (22). Gonadotropins are regulated with cyclic adenosine 3',5'-monophosphate (cAMP) and subsequent activation of

Table. Meiotic competence of dog oocytes after in vitro oocyte maturation for 72 h in the presence of different concentrations (0.05–0.1 IU/mL) of pituitary (p) and recombinant human (rh) gonadotropins.

Groups	n	Developmental stages and rates % (cell numbers)					
		GV	GVBD	MI	MII	MI + MII	UDNM
Control (No gonadotropins)	165	10.30 [b] (17/165)	30.30 (50/165)	33.94 [b] (56/165)	4.24 (7/165)	38.18 [b] (63/165)	21.21 (35/165)
0.05 IU/mL pFSH + pLH	166	16.87 [ab] (28/166)	19.28 (32/166)	46.39 [a] (77/166)	6.02 (10/166)	52.41 [a] (87/166)	11.45 (19/166)
0.1 IU/mL pFSH + pLH	187	11.77 [b] (22/187)	25.67 (48/187)	48.13 [a] (90/187)	1.60 (3/187)	49.73 [a] (93/187)	12.83 (24/187)
0.05 IU/mL rhFSH + rhLH	165	12.73 [b] (21/165)	28.49 (47/165)	39.40 [ab] (65/165)	4.24 (7/165)	43.64 [ab] (72/165)	15.15 (25/165)
0.1 IU/mL rhFSH + rhLH	162	20.99 [a] (34/162)	29.63 (48/162)	29.63 [b] (48/162)	4.94 (8/162)	34.57 [b] (56/162)	14.82 (24/162)

[a, b, c] Values with different superscripts in the same column are significantly different ($P < 0.05$).
pFSH: Pituitary follicle stimulating hormone; pLH: pituitary luteinizing hormone.
rhFSH: Recombinant human follicle stimulating hormone; rhLH: recombinant human luteinizing hormone.
GV: Germinal vesicle; GVBD: germinal vesicle breakdown; MI: metaphase I; MII: metaphase II; UDNM: undetermined nuclear material.

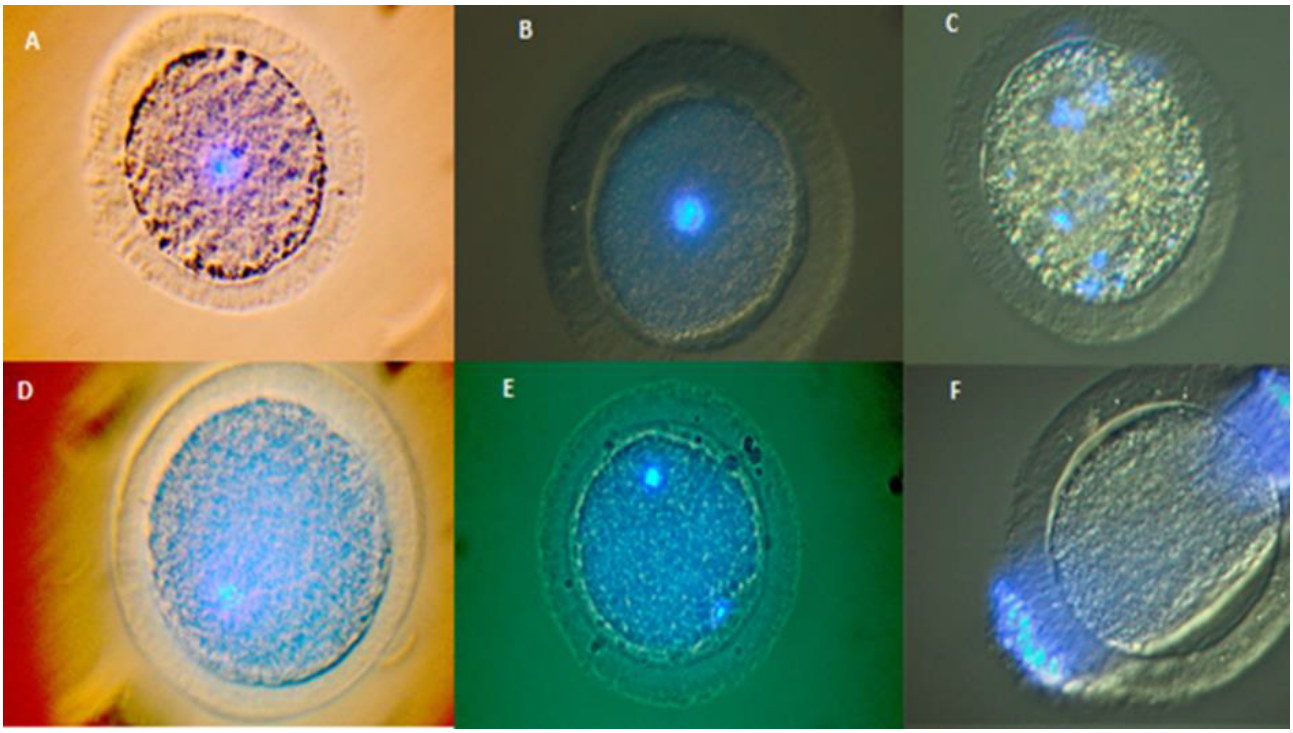

Figure 1. Canine oocytes stained with Hoechst 33342 and visualized under an epifluorescence microscope. A) Germinal vesicle; B) germinal vesicle breakdown; C) degenerated; D) metaphase I; E) metaphase II; F) undetermined nuclear material (UDNM) (200× magnification).

mitogen-activated protein kinase (MAPK) (23). Although the meiotic resumption is stimulated by gonadotropins in mammals, its mechanism is still not fully understood. It has been demonstrated that human recombinant gonadotropins stimulate ovulation, maturation, and steroidogenesis in rats (24). Moreover, rhFSH alone or combined with rhLH in the presence of estrogens could have inducement efficacy on progression of the meiotic cycle of sheep oocytes (18). Ability of rhFSH and rhLH to cause complete in vitro maturation and embryonic development has been demonstrated for the first time in bovine oocytes (20). However, previous studies reported similar results for recombinant gonadotropins on human embryonic development (25). It was stated that rhFSH at doses of 0.1–1.0 IU/mL was appropriate to stimulate in vitro oocyte maturation and subsequent embryo development in domestic cat (19). In contrast, the present study indicates that the highest maturation rates were induced using pituitary gonadotropin (pFSH + pLH), while the lowest maturation rates were found in the control and 1.0 IU recombinant human gonadotropin groups (38% and 34%, respectively). Furthermore, our findings were in contrast with other studies that stated that gonadotropins are not required for in vitro meiosis in cultured mammalian oocytes (26). The presented data showed that the physiological processes and in vitro environmental requirement of dog oocytes differ from other mammalian oocytes.

Contamination of biologically derived hormones with minor components may play an important role on in vitro oocyte quality and viability (20). Previously, we found that applying recombinant gonadotropin in cats at 0.5 IU/mL was superior to pituitary hormones in inducing maturation (44.62%) (27). In this study the 1.0 IU human recombinant gonadotropin group had dramatically decreased maturation rates of dog oocytes. Although the 0.5 IU human recombinant gonadotropin results (43.64%) were statistically similar to 0.5 IU and 1.0 IU pituitary results (52.41% and 49.73%), the results from 1.0 IU recombinant gonadotropins (34.57%) were lower than even those of the control group (38.14%). These findings indicated that dog and cat oocytes might require other factors, such as pituitary-based thyroid stimulating hormone (TSH) or other biological components, during the long-term maturation process. It is known that FSH has a positive effect on the expansion of cumulus cells as well as a positive correlation with the meiotic development ability in mammalian oocytes (28). In contrast to this knowledge, it is declared that the IVM rate of canine oocytes is negatively correlated with cumulus expansion rates. Moreover, the presence of gonadotropins for the culture period has a negative effect on the nuclear maturation rates (29). Although bovine somatotropin increased the rate of blue fox oocytes reaching the MII stage, no effect on nuclear maturation was observed in dogs (30). These findings demonstrate that there is some divergence between canines and other mammals and even among canid species in the response of their oocytes to hormone supplementation. It has been concluded that 0.5 IU recombinant human gonadotropin could give results similar to those of biologically derived hormones and thus can be used instead of biologically derived hormones for in vitro oocyte maturation in dogs.

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