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Structural Changes of Zona Pellucida Surface of Immature, *In vivo* and *In vitro* Matured Canine Oocytes Using Scanning Electron Microscopy

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Abstract

Zona pellucida (ZP), a primarily representative coat of mammalian egg and embryo, has an extremely heterogeneous morphology during different developmental stages. The objective of the present study was to compare the morphological changes of the ZP surface of immature, *in vitro* and *in vivo* matured canine oocytes by using scanning electron microscopy (SEM). Canine ovaries were collected from local veterinary hospitals to recover immature oocytes. The ovaries were sliced and the released cumulus oocyte complexes (COCs) were washed with TL-HEPES. The selected COCs were randomly divided into two groups, first group was processed immediately at immature state and the second group was processed 72 h after *in vitro* maturation, and compared with *in vivo* derived oocytes. Oocytes were fixed, critical point dried and examined under SEM. The diameters of oocyte and outer holes of the ZP were measured on a total of 249 oocytes; the results were analyzed using One-way ANOVA. Our results showed that, the diameter of immature oocytes significantly differed ($p < 0.05$) from that of *in vivo* matured oocytes (79.60 ± 0.77 μm vs. 101.46 ± 1.07 μm, respectively). Similarly, a significant difference ($p < 0.05$) in the diameters between those of *in vitro* and *in vivo* matured oocytes were found (79.51 ± 2.36 μm vs. 101.46 ± 1.07 μm, respectively). Moreover, the diameters of the outer holes of the ZP were significantly ($p < 0.05$) larger in *in vivo* matured (1.48 ± 0.42 μm) than *in vitro* matured for 72 and immature oocytes (1.10 ± 0.16 and 0.43 ± 0.12 μm, respectively). Taken together, these data indicates that the ZP surface is related to oocyte maturity in canine.

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INTRODUCTION

In vitro maturation (IVM) of cumulus oocyte complexes (COCs) is an extremely important step in assisted reproductive technology (ART) for the successful fertilization and further embryo development (Mesalam et al. 2017a, Mesalam et al. 2017b). Moreover, oocyte quality is a key factor for successful embryo development in ART (Mesalam et al. 2018). The efficiency of *in vitro* oocyte maturation in domestic dog is very low compared to that of other mammalian species (Luvoni et al. 2005). In canine species, is well known that embryonic development depends on oocyte competence, involves both cytoplasm and nucleus, which progressively acquired by the oocyte during the process of maturation (Reynaud et al. 2006). The acquisition of competence is a highly coordinated process that includes morphological, ultrastructural, and transcriptional changes of the cytoplasmic and nuclear compartment of the oocyte (Royere 2006). Canine oocyte ovulated at an immature germinal vesicle (GV) stage and maturation occurs in oviduct after 56 - 72 h (Reynaud et al. 2012). The mechanisms that regulate canine oocyte cytoplasmic and nuclear maturation are not fully understood. Many early ultrastructural changes of developing oocytes in the dog are similar to other mammalian oocytes (Tesoriero 1982). However, the dog oocyte is distinguished by the presence of large amounts of lipid yolk material that gives a dark and homogeneous appearance to the oocyte (Guraya 1965).

The plasma membrane of mammalian and non-mammalian eggs is surrounded by at least one extracellular coat (Wassarman 2008). The importance of the zona pellucida with regard to fertilization is well established and widely known (Herrler and Beier 2000). The ZP is an extracellular matrix that acts as a protective coat enveloping oocyte and embryo until zona hatching prior to implantation (Kolbe and Holtz 2005) and plays important functions during gamete interaction, fertilization and early embryonic development (Clark 2010). Mammalian ZP is a fibrous network composed of three glycoproteins, ZPA, ZPB and ZPC, that are responsible for species-restricted binding of sperm to unfertilized eggs (Wassarman 2008). Sperm attachment to ZP triggering the acrosome reaction and zona glycoproteins supporting this binding during sperm penetration (Bleil and Wassarman 1983). Only competent ZP glycoproteins ZPB and/or ZPC are able to mediate sperm attachment and binding (Michelmann et al. 2007). In mouse the growing oocyte is the only source of ZP glycoproteins, while in dogs these proteins are expressed in both the oocyte and granulosa cells in a sequential manner during folliculogenesis (De los Reyes et al. 2009).

The arrangement of zona architecture during oocyte maturation appears to be different among species (De los Reyes et al. 2009). It is unclear whether the zona pellucida (ZP) also has to undergo maturation to become fully competent to allow interaction with spermatozoa and adequately protect the growing embryo (Michelmann et al. 2007). Recently, the molecular mechanisms of sperm-oocyte interactions gained greater insight. We hypothesized that, studying the ultra-structure of ZP using scanning electron microscopy at different stages of maturation may help in understanding the mechanism of sperm-oocyte interactions. The objective of this study was to compare the morphological changes of the ZP surface of immature, *in vitro* and *in vivo* matured canine oocytes by using scanning electron microscopy (SEM).

MATERIALS AND METHODS

1. Chemicals

Unless otherwise indicated, all of chemicals and media used in the present study were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2. Ethics statement

All of the methods and experimental procedures were conducted according to the approved (Approval ID: GAR-110502-X0017) guidelines and regulations by the institutional animal care and use committee (IACUC) of the division of applied life sciences, department of animal science at Gyeongsang National University, Republic of Korea.

3. Collection of canine ovaries and *in vitro* maturation of cumulus-oocyte complexes

Ovaries were retrieved from female dogs (n = 14) undergoing an ovariohysterectomy at local veterinary hospitals, placed in D-PBS supplemented with 1% penicillin-streptomycin and transported to the laboratory within 2 h. Ovaries were put in TCM-199 medium with 25 mM HEPES supplemented with 0.1% bovine serum albumin (BSA) and 1% penicillin-streptomycin at 38.5ºC and the ovarian cortex was repeatedly sliced. Perfectly spherical oocytes with a uniform, smooth and dark cytoplasm were selected to be matured *in vitro* (n=203). Oocytes were cultured in TCM-199 supplemented with 1 μg/mL of estradiol-17ß, 10 μg/mL of FSH, 0.6 mM of cysteine, 0.2 mM of sodium-pyruvate, 10% FBS and 1% penicillin-streptomycin. Maturation was performed by culturing

approximately 40-50 COCs in 500 μL of maturation medium in 4-well dish (Thermo Fisher Scientific, Waltham, MA, USA) for 72 h at 5% CO_2 and 38.5ºC.

4. Collection of *in vivo* matured oocytes

In vivo matured oocytes were collected from six female dogs as previously described (Jang et al. 2007) with slight modification. In brief, blood samples were collected from dogs and serum progesterone (P_4) concentrations were determined by radioimmunoassay (Neodin, Seoul, Republic of Korea). The day that serum progesterone concentration reached 4.0 - 7.5 ng/mL was considered the day of ovulation. Three days after ovulation, *in vivo* matured oocytes were retrieved by laparotomy under anesthesia (1 mg/kg xylazine and 2% isoflurane for maintain of anesthesia). A total of 46 COCs were collected by flushing uterine tubes with 10 mL of TCM-199 medium with 25 mM HEPES supplemented with 10% FBS.

5. Assessment of oocyte nuclear maturation

Nuclear status assessments were carried out as previously described (Songsasen et al. 2012). In brief, oocytes were fixed by placement into wells containing 1:3 acetic acid:ethanol solution for 48 h, then stained using aceto-orcein (1% [w/v] orcein in 45% [v/v] acetic acid) and washed in aceto-glycerol (1:1:3 glycerol:acetic acid:distilled water). Nuclear status was evaluated under light microscopy.

6. Scanning electron microscopy

Oocytes were prepared for scanning electron microscopy (SEM) as previously described (Moreira da Silva and Metelo 2005). Briefly oocytes were placed in fixation medium (2.5% glutaraldehyde [v/v] and 0.1 M sodium cacodylate buffer) for 2 h at 4°C. Thereafter, they were washed with 0.1 M sodium cacodylate buffer containing 0.5 M saccarose and kept in this buffer for 2 h at 4°C, followed by washing in distilled water for 5 min. Oocytes were then dehydrated with increasing concentrations of ethanol and then post-fixed in 1% (w/v) osmium tetroxide for 1 - 2 h at room temperature, dried and mounted onto a gold grids. Oocytes were examined with a JEOL JSM 6301F SEM (Jeol, Peabody, MA, USA) using magnification of 5000 X. Photomicrographs were taken, images were printed and ZP pores were counted in areas of 12 × 8 μm (5000 X). The diameter of each pore was also determined.

7. Statistical analysis

Statistical analyses were performed using SPSS software v.18.0 (IBM Corp., Armonk, NY, USA). One-way ANOVA was performed to assess differences among groups. The data are presented as the mean ± SD. Differences between means were considered to be significant at $p < 0.05$.

RESULTS

1. Assessment of oocyte nuclear maturation

The nuclear maturation of oocytes was evaluated by the presence of an extruded first polar body using aceto-orcein staining. We found that a significantly ($p < 0.05$) higher percentage of oocytes progressed to MII stage in the *in vivo* matured oocytes compared to *in vitro* matured oocytes (21.7 ± 3.5% vs. 4.9 ± 4.6%, respectively, Table 1).

2. Comparison of oocyte diameter and ZP pore size

The SEM data showed a rough fibrous network with elliptical and spherical holes in the surface of the ZP in all groups (Figure 1). The mean diameters of oocyte did not significantly differed among immature (79.60 ± 0.77 μm) and *in vitro* matured oocytes (79.51 ± 2.36 μm) (Table 2). However, our observations demonstrated that the diameters of immature oocytes significantly differed (*p*

Table 1. Nuclear maturation status of in vivo ovulated oocytes and in vitro matured oocytes for 72 h as determined by aceto-orcein staining

Source of oocytes	No. of oocytes	No. oocytes developed to (% ± SD)				
		GV	GVBD	MⅠ	MⅡ	Unclassified
In vivo	46†	12 (26.1 ± 3.6)^b	8 (17.4 ± 3.0)^b	0	10 (21.7 ± 3.5)^a	16 (34.8 ± 6.1)^a
In vitro	203*	72 (35.5 ± 18.3)^a	45 (22.2 ± 13.5)^a	37 (18.2 ± 6.6)	10 (4.9 ± 4.6)^b	39 (19.2 ± 15.3)^b

† Six replicates were performed.

* Fourteen replicates were performed.

Abbreviations: GV: germinal vesicle, GVBD: germinal vesicle breakdown, MⅠ: metaphase Ⅰ, MⅡ: metaphase Ⅱ.

Table 2. Oocyte diameter and zona pellucida pore size of immature, in vitro matured for 72 h and in vivo matured canine oocytes as determined by scanning electron microscopy

Types of oocyte	No. of oocytes analysis	Oocyte diameter (μm)	Pore size (μm)
Immature	20	79.60 ± 0.77[a]	0.43 ± 0.12[a]
In vitro matured MⅡ for 72 h	10	79.51 ± 2.36[a]	1.10 ± 0.16[b]
In vivo matured MⅡ	10	101.46 ± 1.07[b]	1.48 ± 0.42[c]

* Values with different superscripts in the same column were significantly different ($p < 0.05$).

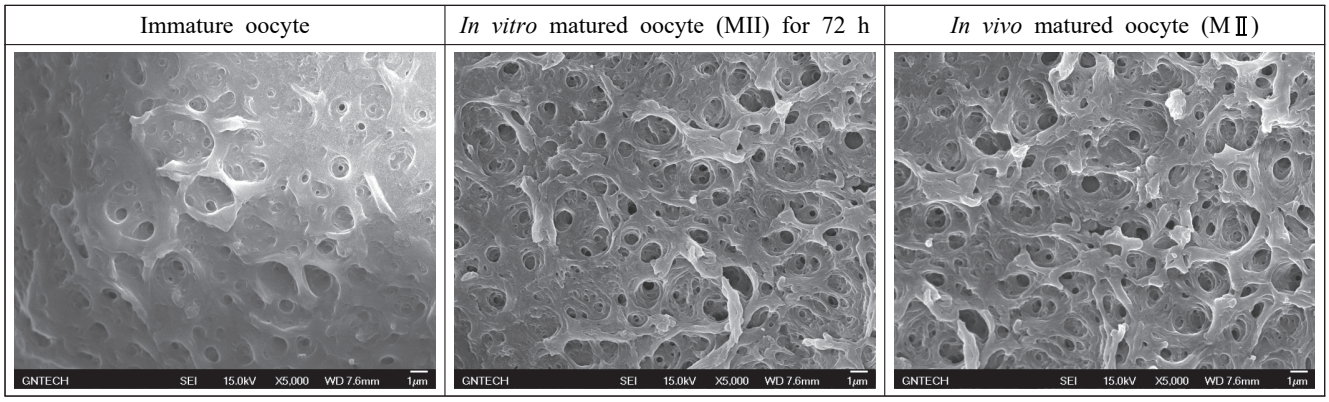

Figure 1. Scanning electron microscopy microphotographs of the zona pellucida of canine oocytes during different states of maturity (Magnification X 5000).

< 0.05) from that of *in vivo* matured oocytes (79.60 ± 0.77 μm vs. 101.46 ± 1.07 μm, respectively, Table 2). Similarly, a significant difference ($p < 0.05$) in the oocyte diameter between *in vitro* and *in vivo* matured oocytes were found (79.51 ± 2.36 μm vs. 101.46 ± 1.07 μm, respectively, Table 2). Additionally, the diameters of the outer holes of the ZP were significantly ($p < 0.05$) different among immature, *in vitro* matured oocytes for 72 and *in vivo* matured oocytes (0.43 ± 0.12, 1.10 ± 0.16 and 1.48 ± 0.42 μm, respectively, Table 2).

DISCUSSION

The ZP has important functions during oocyte maturation and embryo development such as sperm attachment in a species-specific manner, blocking polyspermy after fertilization, facilitating the passage of the embryo through the oviduct, preventing the dispersion of blastomeres during pre-implantation development, and protecting the embryo during early stages of development (Fujiwara et al. 2009, Vanroose et al. 2000). The surface structure of the ZP from oocytes of different mammals, mice, hamsters, pigs and cattle, has been described by several authors (Michelmann et al. 2007). However, fewer studies has been conducted on domestic dog, which could be a valuable model for studying and developing assisted reproduction (Luvoni et al. 2005). Here, we performed comparative analysis of ZP ultrastructure in immature, *in vitro* and *in* vivo matured canine oocytes using SEM.

In contrast to mouse and goat, where the major sizes of pores are associated to the typical pattern of ZP of the fully matured oocytes, bovine oocytes have finer fibrous network of the ZP at the end of maturation, while porcine ZP have more compact and smooth surface after maturation (De los Reyes et al. 2009). It has been reported that small diameter ZP pores is linked to superior oocyte quality as well as to a high rate of blastocyst formation (Santos et al. 2008). *In vivo* matured porcine oocytes showed a mesh-like structure with numerous fenestrations, while *in vitro* matured ones were more compact and smooth, which is indicative of incomplete ZP maturation (Funahashi et al. 2000). Our result showed that the mean pore size of canine ZP were significantly different in immature, *in vitro* matured oocytes for 72 h and *in vivo* matured canine oocytes, with higher size in *in vivo* matured oocytes. Previous study reported that large diameter pores may help to orientate sperm during oocytes penetration (Suzuki et al. 2000).

In conclusion, our results show that structural changes in the ZP surface is related to maturation process in canine oocytes, which could influence sperm binding and penetration during the gamete interaction.

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CONFLICTS OF INTEREST

None of the authors have any conflicts of interest to declare.

AUTHOR CONTRIBUTIONS

Byung-Hyun Choi, Ayman Mesalam, Seok-Hwan Song, Myeong-Don Joo, Ji-Yoon Hwang, Seon-Hwa Oh and Kyeong-Lim Lee performed all of experiment and the analysis of zona pellucida by SEM. Il-Keun Kong designed the experimental protocol, performed the statistical analyses, wrote and edited the manuscript.

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