

Ultrastructural Study of the Canine Zona Pellucida Surface During *In Vitro* Maturation

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Contents

The aim of the present study was to compare the ultrastructure of the surface of the zona pellucida (ZP) of immature and *in vitro* matured dog oocytes using scanning electron microscopy (SEM). Bitch oocytes were collected after ovariohysterectomy; the ovaries were sliced and the released cumulus–oocyte complexes (COCs) were washed with phosphate buffered saline (PBS). The selected COCs were randomly allocated into three groups, two groups were processed after *in vitro* maturation at both 72 and 96 h and a third group was processed immediately at immature state in PBS medium. After that, oocytes were fixed, critical point dried and viewed by using SEM. The diameters of the outer holes of the ZP were measured on a total of 93 oocytes; the results were analyzed with ANOVA. The mean diameters of holes were different between groups ($p < 0.05$): 0.69 ± 0.12 , 1.56 ± 0.19 and $1.42 \pm 0.27 \mu\text{m}$, for immature and *in vitro* matured oocytes for 72 and 96 h, respectively. The difference in the hole sizes between immature and *in vitro* matured canine oocytes indicates that the ZP surface is related to oocyte maturity in canines.

Introduction

The zona pellucida (ZP) is an extracellular matrix that plays important functions during gamete interaction, fertilization and early embryonic development. Zona glycoproteins mediate sperm attachment and binding in a species-specific manner, triggering the acrosome reaction and supporting this binding during sperm penetration (Bleil and Wassarman 1983).

The ZP of mammalian species is a fibrous network, which is composed of three glycoproteins (ZPA, ZPB, ZPC), products of the gene families ZPA, ZPB and ZPC that have a high homology within mammals (Harris et al. 1994). Each of these glycoproteins is an essential structural component of the extracellular coat. Contrary to the mouse, where the growing oocyte is the only source of ZP glycoproteins, in dogs, these proteins are expressed in both the oocyte and granulosa cells in a sequential manner during folliculogenesis (Blackmore et al. 2004).

During maturation, the oocyte undergoes nuclear and cytoplasmic changes, including oocyte investments (vitelline membrane, ZP and cumulus cells). In the course of acquiring these competences, variations in the architecture of the ZP surface have been described in some species (Suzuki et al. 1994; Villamediana et al. 1999; Michelmann et al. 2007); however, the arrangement of zona architecture during oocyte maturation appears to be different among species. The objective of this study was to evaluate morphological changes of the ZP surface of immature and *in vitro* matured bitch

oocytes cultured during two different periods (72 and 96 h) by using scanning electron microscopy (SEM).

Materials and Methods

All the reagents were purchased from Sigma (Sigma Co., St Louis, MO, USA) unless otherwise specified.

Oocyte processing

Cumulus–oocyte complexes (COCs) were obtained from 14 normal bitch ovaries following ovariohysterectomy and selected if they had homogeneous dark cytoplasm with more than three compact cumulus cells layers. The selected COCs were washed in PBS and randomly allocated into three groups: (a) one group was incubated for *in vitro* maturation (IVM) in tissue culture medium (TCM)-199; Earle's salt buffered with 25 mM Hepes (Invitrogen; Carlshead, USA), supplemented with 10% foetal calf serum, 2.5 $\mu\text{l}/\text{ml}$ pyruvic solution (11.2 mg/ml pyruvic acid), 10 IU/ml of human chorionic gonadotrophin and 5 $\mu\text{l}/\text{ml}$ antibiotic solution (12.2 mg/ml penicillin and 20 mg/ml streptomycin) for 72 h; (b) the second group was cultured in the same medium for 96 h (De los Reyes et al. 2005) and (c) the third group was processed immediately at an immature state in PBS medium.

Scanning electron microscopic analysis

Immature and *in vitro* matured oocytes at each culture period were washed in PBS and processed for SEM. After removal of the cumulus cells, oocytes were fixed for 1 h in 2.5% (v/v) glutaraldehyde in a 0.1-M sodium cacodylate buffer, pH 7.4, and post-fixed in 2% osmium tetroxide. Then the oocytes were dehydrated in increasing concentration of acetones, critical point dried, mounted and sputtered with palladium–gold target. The samples were evaluated with a 'LEO' 1420 VP SEM (Carl Zeiss, Oberhofen, Germany). The ZP network surface (hole diameters within an area of $112 \mu^2$ per oocyte) were measured by the 'AUTOCAD 2006' software (Autodesk, Inc. San Francisco, CA, USA) on a total of 93 canine oocytes obtained throughout five replicates: 30 oocytes were evaluated at immature state, 31 after 72 h and 32 after 96 h of culture. Only oocytes showing surface network of the ZP with multiple pores and hollows were used for experiments. The results were analyzed by ANOVA using Tukey's test to determinate the differences $p < 0.05$.

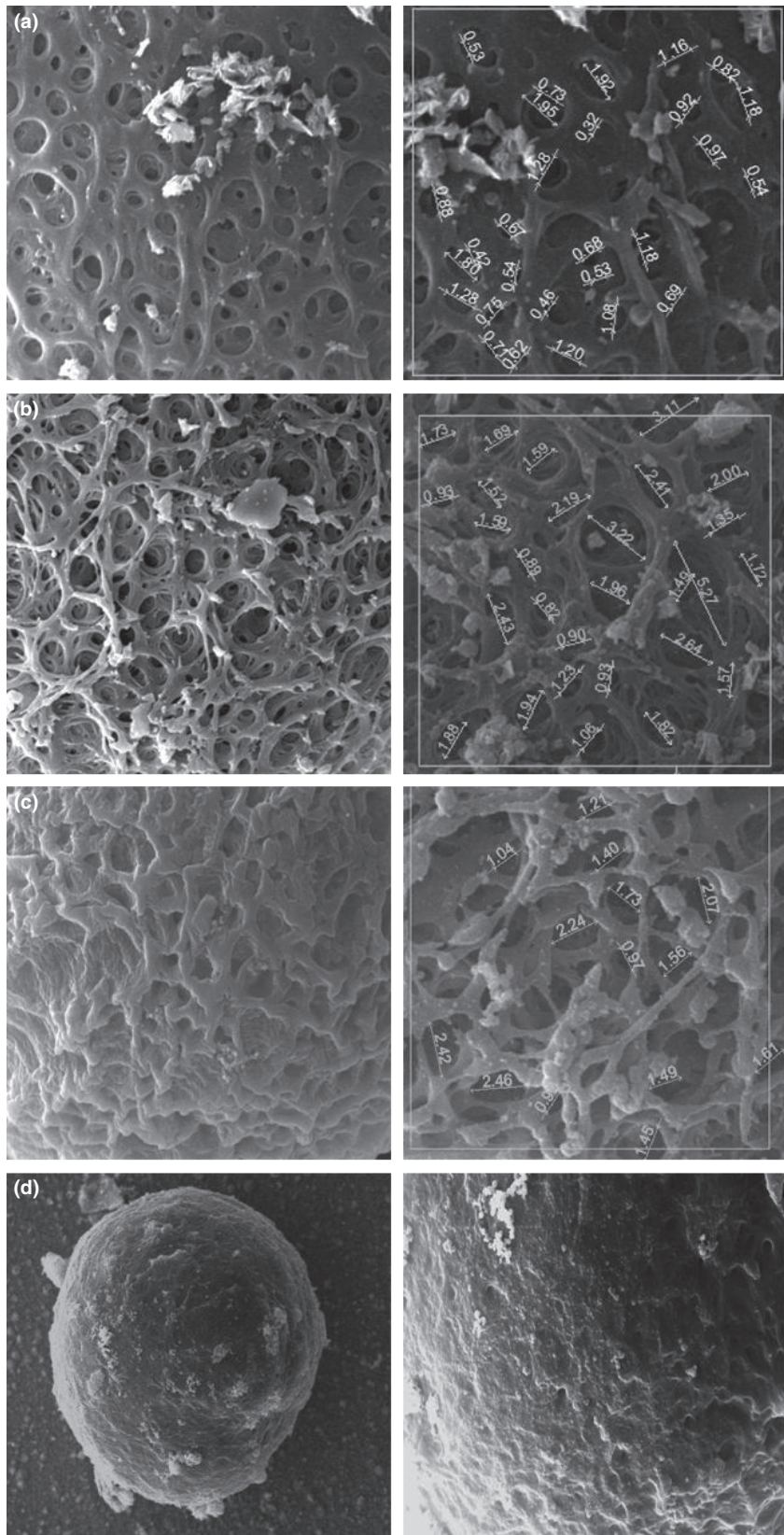


Fig. 1. SEM microphotographs of the ZP of canine oocytes during different states of maturity. Surface characteristics of the ZP of (a) immature oocytes; (b) *in vitro* matured for 72 h; (c) *in vitro* matured for 96 h and (d) degenerated oocyte. (a, b, c: Magnification X 5000 left hand side, with one of the corresponding quadrant digitally amplified used for pore measurements, right hand side. d: magnification X 1500).

Results

The SEM study revealed a rough fibrous network with elliptical and spherical holes in the surface of the ZP of non-degenerating oocytes; however, our observations demonstrated that the zona surface holes of immature

oocytes differed from that of matured oocytes. A significant difference ($p < 0.05$) in their mean diameters between those located in the surface of immature and *in vitro* matured oocytes were found (Fig. 1). Before IVM, the ZP surface showed numerous tight holes

(mean \pm SD diameter $0.69 \pm 0.12 \mu\text{m}$; Fig. 1a), and after culturing, the ZP surface was characterized by large holes (Fig. 1b,c).

A significant difference was also observed between each time period of incubation for IVM ($p < 0.05$). Oocytes cultured for 72 h showed wider holes than oocytes cultured for 96 h: mean \pm SD diameter 1.56 ± 0.19 and $1.42 \pm 0.27 \mu\text{m}$, respectively.

A great proportion of degenerating oocytes (Fig. 1d) was also observed in all groups irrespective of maturity. These oocytes were mainly characterized by deformations and amorphous ZP surface without holes.

Discussion

There is still very scarce information on the ultrastructure of canine oocytes; this study demonstrated that the surface net-like structure of the ZP in canine oocytes undergo changes during IVM. These changes in the appearance of the ZP during maturation have been described in other species, although no measurement of the size of holes has been performed before and after maturation, our observations are in agreement with previous studies which have correlated the type of structure of the ZP and the stage of maturity (Familiari et al. 1988; Calafell et al. 1992; Suzuki et al. 1994; Villamediana et al. 1999). The mesh arrangement of the fibrous network observed using SEM showed tight holes in immature oocytes, which tended to be wider after the process of maturation. The major sizes of pores have been previously associated to the typical pattern of ZP of the fully matured mouse (Calafell et al. 1992) and goat (Villamediana et al. 1999) oocytes. In contrast, in bovine oocytes, the fibrous network of the ZP became finer at the end of maturation (Suzuki et al. 1994). In the same way, studies in porcine ZP have reported that maturation causes a more compact and smooth surface (Michelmann et al. 2007). Yet, *in vivo* matured dog oocytes observed with transmission electron microscopy show an outer coat of the ZP with a number of holes in oocytes at second metaphase stage (MII; Viaris de Lesegno et al. 2008), which would be in accordance with our finding.

On the contrary, it has also been suggested that a ZP with an amorphous smooth surface corresponded to degenerating oocytes (Villamediana et al. 1999). Despite that there may be species differences, in the present study, many immature and *in vitro* matured oocytes presented such condition, showing malformations and a compact type appearance with no pores to be measured. Those oocytes were discarded because probably corresponded to degenerating or activating oocytes. The procedures for SEM might cause deleterious effects on some oocytes because a similar proportion of this type of oocytes was observed in immature and *in vitro* matured groups; furthermore, the oocytes were obtained by slicing the ovary, and although it is supposed that all of them were at germinal vesicle stage (GV), oocytes recovered from ovaries may be in different degree of atresia.

The topography of the ZP surface may contribute to the initial interplay between male and female gametes; functional ability of the ZP to bind spermatozoa has

been closely related to its morphological appearance (Strom-Holt et al. 2000). It has been hypothesized that the wide mesh-like arrangement of the ZP after maturation could assist spermatozoa in becoming appropriately oriented to fertilize the oocyte (Villamediana et al. 1999). Our previous findings have shown that *in vitro* matured oocytes showed higher penetration rates than did immature oocytes irrespective of sperm treatment (De los Reyes et al. 2009), which could be in agreement with the different ZP appearances in both types of oocytes (immature and *in vitro* matured) reported in the present work.

Comparing the two culture pounds for IVM, the zona structure of oocytes cultured for 96 h showed smaller holes than those for 72 h. We have previously found a higher percentage of MII stage in oocytes maturing by 96 h in comparison to 72 h, with the same culture condition used in these experiments (De los Reyes et al. 2005); however, ongoing studies at our laboratory have shown that, prolonging the IVM period by 96 h decreased significantly the sperm penetration to the ZP (data not shown). Otoi et al. (2004) reported that, although more dog oocytes could reach MII when they were cultured for a long period, the optimum maturation time seems to be earlier than that, based on embryonic development after IVF. In mice, the structure of the ZP changes with the age of the oocyte (Longo 1981). Thus, the morphological differences in the porous surface between the ZP of each type of oocytes (72 vs 96 h) may be related to aging process, increasing the fusion of layers of the network after prolonged time of incubation, which in turns may have an effect on fertilization.

In conclusion, these results show that structural changes in the ZP surface occur during IVM of canine oocytes; this mesh-like arrangement of the ZP after maturation could influence sperm binding and penetration during the gamete interaction.

Acknowledgement

This research was supported by Grant FONDECYT 1080618.

Author contributions

MDLR designed the study, supervised the experiments and drafted the paper; JH collected ovaries and processed the oocytes sampled; JP participated in the implementation of the scanning electron microscopy protocols, analyzed the data and performed the statistical analysis. All authors read, revised and approved the final manuscript.

Conflicts of interest

The authors have declared no conflicts of interest.

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Submitted: 30 June 2008

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