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CHARACTERIZATION OF IMMATURE SHEEP OOCYTES AND ZONA PELLUCIDA MORPHOLOGY USING SCANNING ELECTRON MICROSCOPE (SEM)

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ABSTRACT

The aim of the present work was to study the differences among different categories of immature sheep oocytes. Oocytes were divided into three categories: excellent, good and fair oocytes. Results showed that cumulus cells were close to each other and zona pellucida in all the three oocytes categories. All oocytes categories with cumulus cells exhibited an irregular shape, mucous material inter-granular cells, low number of small droplets and minutes tapes on the cell surface. Good oocytes category was characterized by the presence of a belt surrounding the oocytes. Moreover, there are large numbers of small holes on the ZP surface in all categories. The good oocytes also contained a large number of big holes. In contrast, fair and excellent oocytes have low number of large holes in the ZP surface. In conclusion, there are several differences between oocytes categories in the morphology of oocytes and ZP characteristics. This study recommends using excellent and good oocytes in the *in vitro* embryo production for quality of the outer surface and integrity of zona pellucida than fair oocytes.

Key words: Immature oocytes, SEM, Sheep, Zona pellucida.

INTRODUCTION

The numbers of sheep can be increased through improved reproductive efficiency of the flock and this reproductive efficiency can be attained through the manipulation of the reproductive activities and technologies (Zhu *et al.*, 2001; Mahammadpour, 2007). Determination of oocyte quality is mostly based on the layers of cumulus cells and cytoplasm or cytoplasm uniformity and colors. This requires considerable experience to better identify oocyte quality because of the intense subjectivity involved (Gordon, 2003). The mammalian zona pellucida (ZP) is an extracellular coat that surrounds growing oocytes, ovulated oocytes, and early embryos. During the initial oocyte growth in secondary follicles, the zona pellucida is secreted by the developing oocyte and surrounding follicular cells as extra-cellular patches that further coalesce into a uniform layer (Dunbar *et al.*, 1994). The ZP has several functions such as binding spermatozoa in a species-specific manner, blocking polyspermy, preventing the dispersion of blastomeres during preimplantation development, facilitating the passage of the embryo through the oviduct, and protecting the embryo during early stages of development (Epifano and Dean, 1994). Riddell *et al.* (1993) demonstrated by SEM that the external surfaces of the ZP of immature oocytes are very irregular, with uneven distribution of numerous pores, crevices, and projections. Recently, the surface of the bovine ZP of immature oocytes, *in vitro*-matured oocytes, and *in vitro*-inseminated oocytes was studied by Suzuki and coworkers (Suzuki *et al.*, 1994a, b; Suzuki *et al.*, 1996). They found that the fine structure of the ZP of immature oocytes characterized by a network with numerous wide meshes and deep holes. Variations in the architecture of the ZP exist between species. When the porous surface is compared among species, the largest pores are observed in the ZP of the rabbit and the cat, the smallest in the cow. The pores are largest at the outer surface of the ZP but decrease in size centripetally

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(Nikas *et al.*, 1994). The smallest size of the fibers composing the zona network is found in the cow and the opossum as reported by (Geert *et al.*, 2000).

Ultrastructural studies of the oocyte during *in vitro* maturation in different mammalian species cattle (Hyttel *et al.*, 1997), camel (Kafi *et al.*, 2005) have resulted in a better understanding of the biology of the oocyte and, as a consequence, improvements in IVM and IVF. Little information is available about the study of oocytes before *in vitro* maturation using scanning electron microscope. Therefore, scanning electron microscope analysis was used throughout this study to examine the ultrastructural characteristics of immature sheep oocytes morphology and zona pellucida according to different grades before *in vitro* maturation.

MATERIALS AND METHODS

Collection of ovaries: Sheep ovaries were collected from slaughtered females from the slaughter house and transported to the laboratory in insulated thermos flask containing phosphate buffered saline supplemented with gentamycin (0.5%), streptomycin (50 mg/ml) and penicillin (60 mg/ml) at 35–37°C within 2–3 h.

Collection of immature oocytes: Cumulus oocyte complexes (COCs) were obtained by aspiration of follicles (2 – 8 mm in diameter) from ovaries using a 10 ml sterile syringe and an 18 G disposable needle. After aspiration, COCs were washed in Earl's tissue culture medium 199 (TCM199; Sigma M4530) supplemented with 10% fetal bovine serum (FBS, Sigma F4135). Recovered COCs were categorized depending upon the homogeneity of cytoplasm and number of cumulus layers as described previously by Blondin and Sirard (1995). The aspirated COCs from follicles were divided into three categories as follows:-

1- Category 1 (C1): COCs are completely invested with many layers (≥5 layers) of cumulus cells.

2- Category 2 (C2): COCs are completely invested with lesser number of layers (≤ 3 layers) of cumulus cells than C1.

3- Category 3 (C3): Denuded oocytes (oocytes without cumulus cell layers) or semi-denuded oocytes (oocytes partially denuded or partially invested with cumulus cells).

Preparation of oocytes for Scanning Electron Microscopic examination: To study the morphology and ultrastucture of zona pellucida (ZP) through scanning electron microscope (SEM), the sheep oocytes were processed according to John (2007). Briefly, oocytes were fixed in 2.5% glutaraldhyde (diluted in buffer) for 3 h at room temperature, washed with buffered solution, fixed again in osmium tetroxide for 1 h, again washed with buffered solution, dehydrated in a graded ethanol series (25%, 50%, 75%, 100% and 100% each for 10 min), air dried, mounted on a specimen stub, coated with a heavy metal, and examined through the SEM. Morphology was examined, interpreted and assessed according to the scheme based on previous works by Adirekthaworn *et al.* (2008) and Hiroyuki *et al.* (1998).

RESULTS AND DISCUSSION

Four hundred and fifty cumulus oocytes complexes were collected from slaughtered female sheep ovaries and divided into three groups according to the homogeneity of cytoplasm and number of cumulus cells (CC) layers. First group was of excellent oocytes that contained more than 4 layers of CC or without cells; second group was of good oocytes that contains from 2 to 4 layers of CC or without; third group was of fair oocytes that included the denuded and semi-denuded oocytes.

The SEM examination of oocytes revealed fair sheep oocytes of group I to be characterized with irregularly shaped ZP (Fig. 1a), with some cavities (Fig. 1a, b), large number of small holes of lesser depth and small number of large holes on the ZP surface (Fig. 1c). ZP surface seemed to have a spongy appearance (Fig. 1d) with CC existing in cluster form around some parts of the ZP (Fig. 1b). There were intergranular cells containing mucous, minutes tapes and growths on the surface of cells as indicated by a red arrow (Fig. 1e). Surface of the CC contained small and deep holes as indicted by black arrow (Fig. 1e) and CC appeared to have weak contact with the ZP of the oocytes but with strong contact between them (Fig. 1b).

The SEM study for good oocytes with CCs revealed them to be like semi-parallegram shape and spongy nature with many large and small holes (Fig. 2a), the contact between CC is not strong and there is a mucous material (cells coated by layer similar mucous) similar soft tissue covered cells and contains few small holes as indicated by red arrow

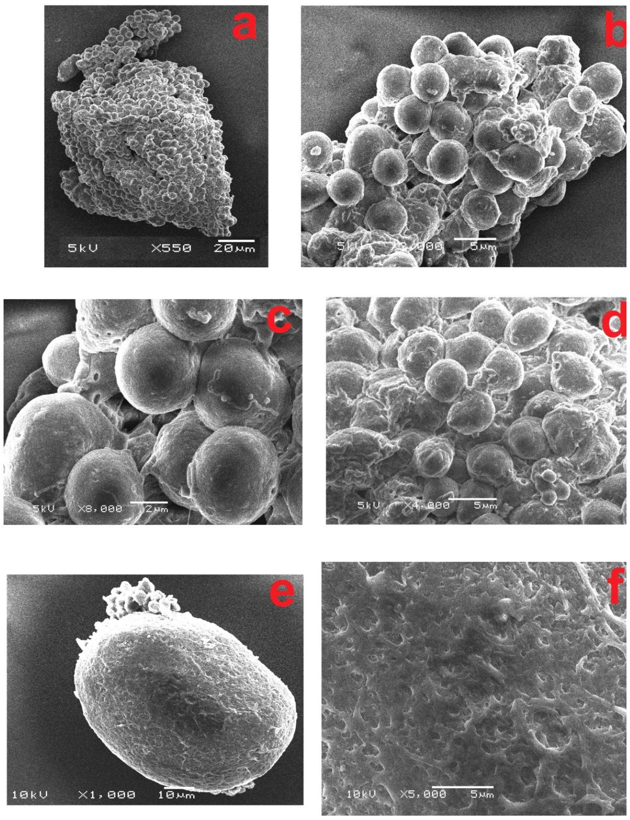

FIG 1: Photographs showing the morphological characteristics and surface properties of zona pellucida of fair sheep oocytes using scanning electron microscope. Minutes taps and growth indicated by a red arrow and small deep holes indicated by black arrow in 1e.

(Fig. 2b) and there is a shape like a belt surround the oocytes as indicated by a black arrow (Fig. 2c). Good oocytes without CC appeared to be spherical in shape with rough texture, low number of droplets as indicated by a black arrow (Fig.2d), numerous large holes and many not deep small holes inside the large holes indicated by red arrow (Fig.2e).

Examination of excellent oocytes (with or without CC) using SEM showed that this grade of oocytes was characterized similar to forms like coral reefs (Fig. 3a) with different sizes of CC (Fig. 3b), low number of small droplets and minutes tapes on the cell surface (Fig. 3c), CC contacted strongly by inter-cellular material like mollusk or gel tissue as showed in the Fig. (3d) all cells coated by this material that indicated by a black arrow. As for excellent oocytes without CC, the SEM revealed the oocytes to be characterized by oval shape and that the surface of ZP was characterized by soft and thick mesh structure (Fig. 3e), small number of large holes and numerous of small holes (Fig. 3f).

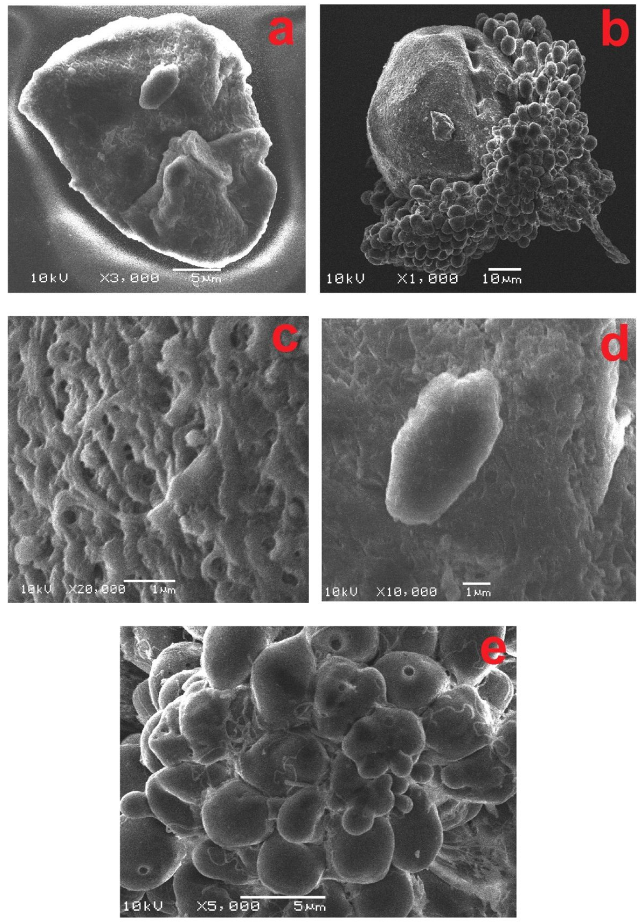

FIG 2: Scanning electron microscope photographs representing the morphological characteristics of good sheep oocytes with cumulus cells and zona pellucida surface characteristics of good oocytes without cumulus cells. Soft tissue and small holes indicated by black and red arrow, respectively as showed in 2b. Belt shape showed in 2c with a black arrow. Droplets in 2d showed by a black arrow. Small holes inside large holes indicated by red and black arrow in 2e.

This study analyzed the morphological characteristics and zona pellucida surface properties of different categories of sheep oocytes (Excellent, good and fair), where there is still very scarce information on the ultrastructure of sheep oocytes. This study demonstrated that there are several differences in sheep oocytes quality among excellent, good and fair. Two principal patterns have been distinguished: 1) a network with a rough and spongy appearance containing numerous pores, and 2) a more compact and melted structure with fewer pores. The same patterns have also been demonstrated in IVM of human oocytes (Geert *et al.*, 2000). These changes in the shape and appearance of the ZP

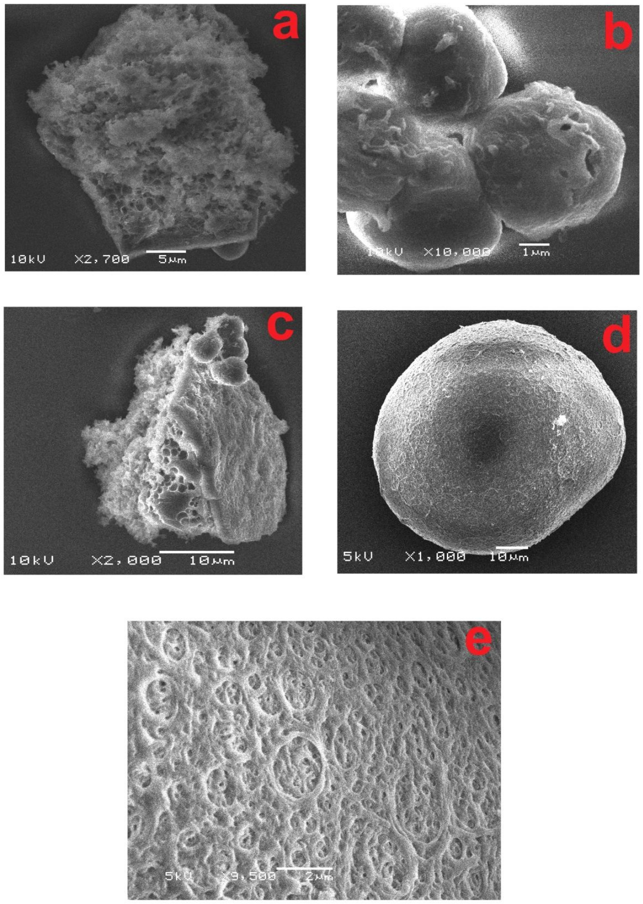

Fig 3: Photographs of scanning electron microscope explaining the excellent sheep oocytes with cumulus cells properties and zona pellucida surface characteristics of excellent sheep oocytes without cumulus cells. Gel tissue in 3d indicated by a black arrow.

surface may be the cause of preference for choosing excellent and good oocytes in the *in vitro* embryo production system. These changes in the appearance of the ZP have been described in other species as a result of some treatments by several authors. The structure of the ZP correlated with the stage of maturity (Suzuki *et al.* 1994a; Reyes, *et al.*, 2009). Observations of the present study are in agreement with Reyes *et al.* (2009), who showed that the fibrous network is in the form of tight holes in immature oocytes. Familiari *et al.* (1992) described a net-like, porous surface mainly in mature oocytes while immature and degenerated oocytes had a compact type as reported in our observations. Riddell *et al.* (1993) demonstrated by SEM that the external surfaces of the ZP of immature oocytes are very irregular, with uneven distribution of numerous pores, crevices, and projections. Recently, the surface of the bovine ZP of immature oocytes, *in vitro*-maturing and *in vitro*-matured (IVM) oocytes, and *in vitro*-inseminated oocytes was studied by Suzuki and coworkers (Suzuki *et al.*, 1994a, b; Suzuki *et al.*, 1996). They found that the fine structure of the ZP of immature oocytes is characterized by a network with numerous wide meshes. Variations in the architecture of the ZP exist between species. When the porous surface compared among species, the largest pores were observed in the ZP of the rabbit and the cat, the smallest in the cow. The pores were largest at the outer surface of the ZP but decreased in size centripetally (Nikas *et al.*, 1994). In general, pore shape varies within and between the zona of the species observed, but many zona exhibited a more elliptical than circular shape. The arrangement of zona pores appeared random in all species except the cat as mentioned by Geert *et al.* (2000). Variation in network thickness has also been observed in our results and in that of others on different species (Suzuki *et al.*, 1994a; Greet *et al.*, 2000).

CONCLUSION

Results obtained by SEM from this study revealed that there are differences between different grades of immature sheep oocytes with regard to the morphology and surface of zona pellucida. These results suggest the use of excellent and good quality oocytes in the production of embryos *in vitro* and do not use fair oocytes, which leads to reduce the cost of *in vitro* embryo production, either in human and animals.

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REFERENCES

Adirekthaworn, A., Tharasanit, T. and Chuesiri, P. (2008). P3 A Scanning Electron Microscopic Analysis of Zona Pellucida of Cryopreserved Porcine Oocytes. *Proceedings 7th Chula. Univ. Vet. Sci. Ann. Con.*, 1 May. pp: 59.

Blondin, P. and Sirard, M.A. (1995). Oocyte and follicular morphology as determining characteristics for developmental competence in bovine oocytes. *Mol. Reprod. Dev.*, **41**: 54-62.

Dunbar, B.S., Avery, S., Lee, V., Prasad, S., Schwahn, D., Schoebel, E., Skinner, S. and Wilkins, B. (1994). The mammalian zona pellucida: its biochemistry, immunochemistry, molecular biology, and development expression. *Reprod. Fertil. Dev.*, **6**:331–347.

Epifano, O. and Dean, J. (1994). Biology and structure of the zona pellucida: a target for immunocontraception. *Reprod. Fertil. Dev.*, **6**: 319–330.

Familiari, G., Nottola, S.A. and Macchiarelli, G. (1992). Human zona pellucida during *in vitro* fertilization: An ultrastructural study using saponin, ruthenium red, and osmium-thiocarbohydrazide. *Mol. Reprod. Dev*., **32:** 51–61.

Geert, V., Hans, N., Ann Van, S., Marie-The´ re 'se, Y., Gerard, C., Patrick, V. and Aart, K. (2000). Structural Aspects of the Zona Pellucida of In Vitro-Produced Bovine Embryos: A Scanning Electron and Confocal Laser Scanning Microscopic Study. *Biol. Reprod*., **62**: 463–469.

Gordon, I. (2003). Laboratory Production of Cattle Embryos. CABI, New York, NY, USA.

Hiroyuki, S., Jyh-Cherng, J.U., John, E. and Xiangzhong, Y. (1998). Surface ultrastructural characteristics of bovine oocytes following heat shock. *J. Reprod. Dev.*, **44**: 345 – 351.

Hyttel, P., Fair, T., Callesen, H. and Greve, T. (1997). Oocyte growth, capacitation and final maturation in cattle. *Theriogenology*, **47:** 23–32.

John, K. (2007). Electron Microscopy Methods and Protocols. Second Edition, [Edited by Humana] Press Inc. Totowa, New Jersey. 07512.

Kafi, M., Mesbah, F., Nili, H. and Khalili, A. (2005). Chronological and ultrastructural changes in camel (Camelus dromedarius) oocytes during in vitro maturation. *Theriogenology*, **63:** 2458–70.

Mahammadpour, A.A. (2007). Comparative histomorphological study of ovary ad ovarian follicles in Iranian Lori-Bakhtiari sheep and native goat. *Pakistan J. Biol. Sci.*, **10:** 673-675.

Nikas, G., Paraschos, T., Psychoyos, A. and Handyside, A. (1994). The zona reaction in human oocytes as seen with scanning microscopy. *Hum. Reprod.*, **9**:2135–2138.

Reyes, M. D., Hetz, J and Palomino, J (2009). Ultrastructural study of the canine zona pellucida surface during *in vitro* maturation. *Reprod Dom Anim*., 44 (Suppl. 2), 247–250.

Riddell, K.P., Stringfellow, D.A., Gray, B.W., Riddell, M.G., Wright, J.C. and Galik, P.K. (1993). Structural and viral association comparisons of bovine zonae pellucidae from follicular oocytes, day-7 embryos and day-7 degenerated ova. *Theriogenology*, **40**:1281–1291.

Suzuki, H., Presicce, G.A. and Yang, X. (1996). Analysis of bovine oocytes matured in vitro vs in vivo by scanning electron microscopy. *Theriogenology*, Abstract 271.

Suzuki, H., Yang, X. and Foote, R.H. (1994a). Surface characteristics and size changes of immature, in vitro matured and in vitro fertilized bovine oocytes. *Theriogenology*, **41**: 307.

Suzuki, H., Yang, X. and Foote, R.H. (1994b) Surface alterations of the bovine oocyte and its investments during and after maturation and fertilization in vitro. *Mol. Reprod. Dev.*, **38**:421–430.

Zhu, S.E., Zeng, S.M., Yu, W.L., Li, S.J., Zhang, Z.C. and Chen, Y.F. (2001). Vitrification of in vivo and in vitro produced ovine blastocysts. *Anim. Biotech.*, **12:** 193-203.