APPENDIX 1

PROCEDURES OF HANDLING AND FREEZING OF SEMEN

Fresh semen

Male Goats

Male, 1.5 to 2.5 year old cross bred goats maintained and reared at the farm of the Institute of Advanced Studies of the University of Malaya were used. They were kept in individual pens, fed with chopped *Pennisetum purpureum* grass and drinking plain water was given *ad libitum*.

ASelection of Female Goats as Teaser

Female, 1.5 to 2.0 year old local goats which were on natural oestrus were used as teasers. The oestrus was confirmed by the presence of slime in the vagina and also by thus being attracted to the male goats.

Collection of Semen

Semen was collected from selected male cross bred goat between 1100 and 1200 hr and was carried out using artifi-

cial vagina (AV) (Fig A.1). Artificial vagina consisted of a rigid hollow cylinder outer casing of 20 cm in length and outside diameter of 5 cm. A thin-walled rubber tube was fitted to the inside of the cylinder and folded over the ends of the casing, forming a jacket. A 5 ml graduated sperm collecting flask was attached to one end of the AV.

Prior to collection of semen, the artificial vagina was cleared and dried to avoid contamination. Warm water at 40-45°C filled inside the jacket. It was then inflated by using hand pump. Exact temperature and the right amount of air pressure at the jacket were necessary to simulate the vagina of a live goat. K-Y lubricating jelly (Johnson & Johnson, UK) was applied to the top part of rubber tube for lubrication.

When the male goat mounted the female, the sheath was grasped and the penis was directed into the artificial vagina. As a result, the animal ejaculated into the artificial vagina (Fig. A.2). The collecting flask was immediately sealed with parafilm and covered with aluminium foil to avoid exposure of the semen to direct sunlight. The semen was kept in a Dewar flask at 39.0°C and transported to the laboratory within the shortest time possible.

Frozen Semen

Sperm Washing Medium for Freezing

The sperm washing medium for freezing was prepared according to Corteel (1976). This involved the mixing of the reagents shown in Table A.1.

Table A.1: Composition of Sperm Washing Medium

Composition	%	Amount (ml)
NaCl	0.9	100
KCl	1.15	4
$CaCl_2$	1.22	3
KH_2PO_4	99.5	0.4
$MgSO_4.7H_2O$	98.0	3.8
$Na_2HPO_4.12H_2O$ (pH 7.40)		12
Glucose anhydride	5.34	4.5

The washing medium can be prepared and stored at 4.0°C up to one week. The medium was rewarmed in water bath at 37.0°C before being used.

Method of Washing of Semen for freezing

4.5 ml washing medium was added to 0.5 ml of semen in a tube. The mixture was then subjected to the procedure similar to that described in 3.6.2.

Preparation of Extender

Tris-citric acid buffer was prepared by dissolving 3.786 g tris (hydroxymethyl)-aminomethan and 2.115 g of citric acid in distilled water and the volume was made to 100 ml. The pH of the buffer was adjusted to 6.75 with the help of 10% citric acid solution. Buffer and freshly collected egg yolk from the freshly collected (i. e. within 10 min) egg were mixed in the ratio of 4:1. The extender was centrifuged at 2000 rpm for 15 min after which the supernatant was taken. Then 6.8% glycerol and 1% fructose were then added to the extender. This extender was kept at 37.0°C in a water bath and ready for use.

Extension and Freezing of Semen

The method used to freeze the semen was as shown by Corteel (1976; 1981). One part of semen and 9 part of extender were mixed (1:10 extension rate). The extended semen was then fitted in French medium straws using micropipette.

After sealing with pollyvinyl alcohol powder, these straws were kept in a refrigerator (4.0°C) for 90 min. This was to lower the temperature of the extended semen from 37.0°C to 5.0°C. The straws were then equilibrated at 5.0°C for another 120 min. The straws were then removed from the refrigerator into the vapours of liquid nitrogen for 9 min after which the straws were dipped in the liquid nitrogen. The straws were then kept in the liquid nitrogen tank for indefinite storage.

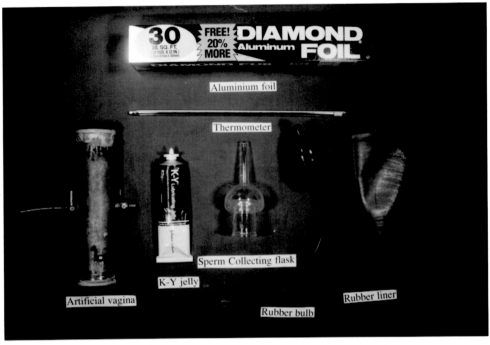


Figure A.1. A set of an artificial vagina used to collect fresh semen. It consists of a rigid hollow cylinder, rubber liner and sperm collecting flask. Rubber bulb was to inflate the rubber liner inside the cylinder. K-Y jelly was to lubricate the top part of rubber tube. Thermometer was to measure the temperature of water inside the jacket. Aluminium foil was used to cover the collecting flask from being exposed to sun light.

Figure A.2. Method of collection of fresh semen from male goat using artificial vagina (AV). A female goat held in stuncheon was used as a teaser.

APPENDIX 2

THE EFFECTS OF FREEZING ON THE ULTRASTRUCTURE OF GOAT SPERM

SUMMARY

The ultrastructure of frozen-thawed goat sperm was compared with those of freshly ejaculated sperm. Comparison was done in terms of the morphology of the head, the presence or absence as well as substructure of the acrosome, the length and internal structure of the neck and different regions of the tail. The study has revealed that there was no aparent differences in ultrastructure between the frozen-thawed and the freshly ejaculated sperm.

INTRODUCTION

The success for the routine IVF-ET procedures very much depend upon the continuous supply of a large number of high quality sperms. This was however, limited by the lack of good quality male goats and the unavailability of females on oestrus at the time when they are needed. In view of this, freezing the freshly ejaculated sperm from selected breed in liquid nitrogen is needed. Although the use of goat frozen sperm for the ET has revealed varying degree of success (Armstrong and Evans, 1983), information regarding the ultrastructure of this sperm is not available. This

information is important to ascertain whether freezing has any effect on the ultramorphology of the sperm since this could affect their fertilizability. Therefore, the intention of this study was to compare the ultrastructure of frozen-thawed goat sperm with those of freshly ejaculated one.

MATERIALS AND METHODS

Collection, Freezing and Treatment of Semen

Semen was collected from male goat using artificial vagina as described in Appendix 1 In the laboratory the spermatozoa were washed with washing medium, also as described before (Appendix 1). Frozen semen was prepared according to the methods described in Appendix 1.

Electron Microscopy

This study was partly done in the ABEL of National University of Malaysia and partly at the Institute of Advanced Studies, University of Malaya. At ABEL, after thawing in a water bath at 37.0°C, spermatozoa were washed as before for the freshly ejaculated sperm. Both samples were taken to Institute of Adsvanced Studies and subjected to transmission electron microscopy (TEM) for morphological analyses. The sperms were initially centrifuged prior to

fixation. The pellet was primarily fixed in 3% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4), post-fixed in 1% (w/v) osmium tetroxide in 0.1 M sodium cacodylate buffer (pH 7.4) and stained with 2% uranyl acetate in 0.005% acetic acid for 15 min at room temperature. Dehydration in graded ethanol series was followed by two changes in propylene oxide and embedded in Mollenhauer's Epon-araldite resin (Mollenhaeur, 1964). Ultrathin sections were stained with uranyl acetate and lead citrate prior to examination in a Philips CM12 transmission electron microscope operated at 80 kV.

RESULTS

Sperm head

The head of both freshly ejaculated and frozen-thawed sperm was elongated and consisted of an electron dense nucleus with a pointed tip (Fig. A.3, A.4 and A.5), a less electron-dense acrosome, bounded by an inner and outer acrosomal membranes, with a bulbous tip (Fig. A.3 and A.4) and extended down two-thirds of the length of the nucleus (Fig. A.6 and A.7). The basal one-third of the nucleus was loosely surrounded by an electron dense postacrosomal sheath (Fig. A.4 and 6.5) while the whole head, 6.5-8.5 μm long, was enveloped by an undulating plasma membrane (Fig. A.3), although it was

occasionally not observed in frozen-thawed sperm (Fig. A.4 and A.5). Occasionally, the frozen-thawed sperm was observed to lack any acrosomal material (Fig. A.7).

Sperm tail

In both freshly ejaculated and frozen-thawed sperm, the tail has a mid-piece (Fig. A.6; A.7; A.8), 0.6-1.2 μm in diameter which consisted of tail fibres and microtubules, surrounded by mitochondria sheath, and a principal piece (Fig. A.9), 0.2-0.6 μm in diameter, which comprised a similar arrangements of fibres and microtubules but the mitochondria sheath was replaced by a fibrous sheath. Throughout the length of the tail, the microtubules consisted of nine axoneme doublets arranged in a ring with a central pair. Each axoneme doublet in the mid-piece has a corresponding electron-dense outer fibre which lies towards the inside of the mitochondria.

DISCUSSION

Within the present preparation, it was difficult to section throughout the length of the sperm. The ultrastructure of morphology of the sperm head, and not the sperm tail, is a key parameter for assessing the sperm fertilizing competence in vitro (Mashiach *et al.*, 1992). The present

study has shown that the head of both freshly ejaculated and frozen-thawed sperm was elongated. Elongated head of frozen-thawed bovine sperm has also been reported (Parrish *et al.*, 1988). Regarding sperm acrosome, Austin and Short (1982) noted that the exact shape of the acrosome is species dependent. Up to this date, apparently there was no similar study reported in goat. In the present study, it was found that there was a less-dense acrosome, bounded by an inner and outer acrosome membranes in both groups of sperms.

The main function of tail is for sperm motility. The high capacity of metabolism of the mid-piece of the tail is reflected by the presence of mitochondrial helix. The presence of microtubules consisted of nine axoneme doubles arranged in a ring with a central pair in both groups of sperms suggest that there was no impairment in sliding movement of microtubules and bending motion of the sperm. In the bovine species, the frozen-thawed and freshly collected semen provided similar rates of penetration following IVF (Pavlock *et al.*, 1988). Therefore, from the present ultrastructural study of goat sperm morphology, it can be assumed that freezing does not impair the fertilizability of sperm of this animal.

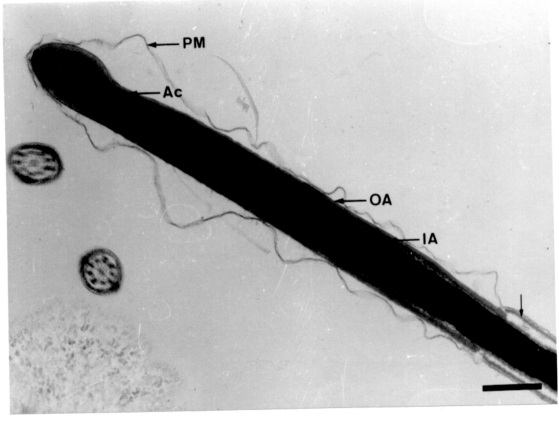


Figure A. 3. Longitudinal section (LS) of part of the head of freshly-ejaculated sperm showing undulating plasma membrane (PM), inner (IA) and outer acrosomal membranes (OA), acrosome (Ac) with a bulbous tip, nucleus (N) and postacrosomal sheath (arrow). Scale bar: 0.5 μm.

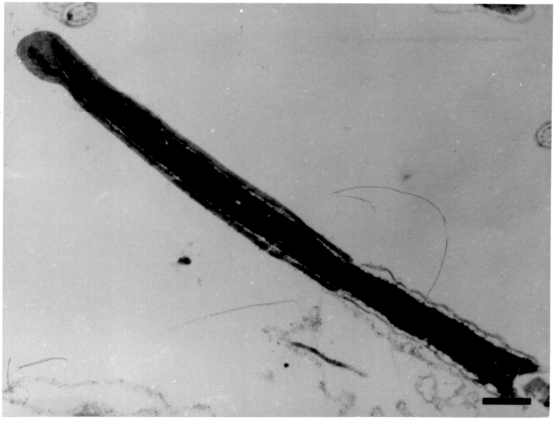


Figure A.4. Longitudinal section (LS) of head of the frozen-thawed sperm showing, occasionally, the absence of plasma membrane. Scale bar: 0.5 μm

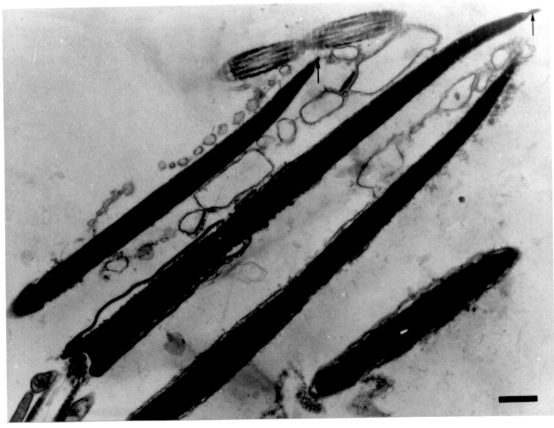


Figure A.5. Longitudinal section (LS) of several heads of frozen-thawed sperms showing, occasionally, the absence of plasma membrane and acrosomal contents. Note the presence of subacrosomal space (arrow). Scale bar: 0.5 μm.

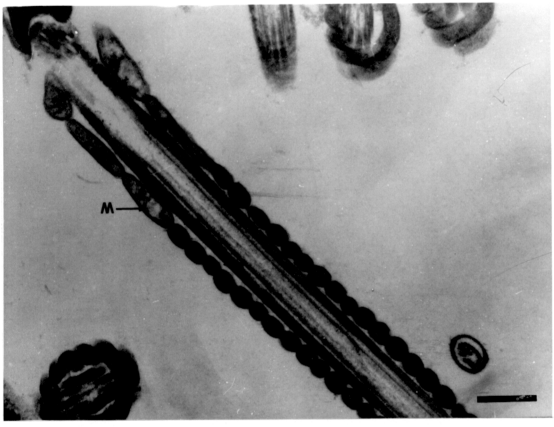


Figure A.6. Longitudinal section (LS) of part of mid-piece of the tail of freshly-ejaculated sperms showing mitochondrial sheath (M) and tail fibres/microtubules (arrow). Scale bar: 0.5 μm

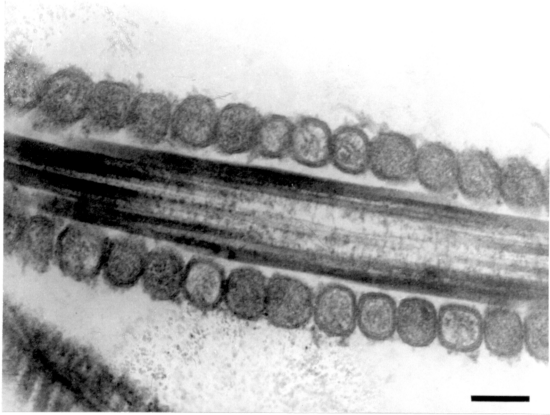


Figure A.7. Longitudinal section (LS) of part of mid-piece of the tail of frozen-thawed sperms showing mitochondrial sheath and tail fibres/microtubules. Scale bar: 0.2 μm

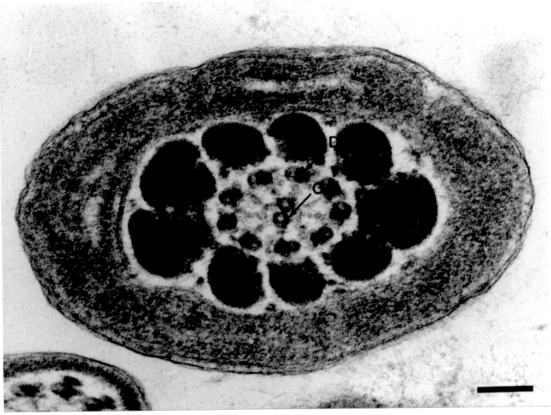


Figure A.8. Transverse section (TS) of mid-piece of the tail of freshly-ejaculated sperms showing mitochondrial sheath (M), 9 outer dense fibres (F), 9 axoneme doublets (D) and central pair (C). Scale bar: 0.1 μm

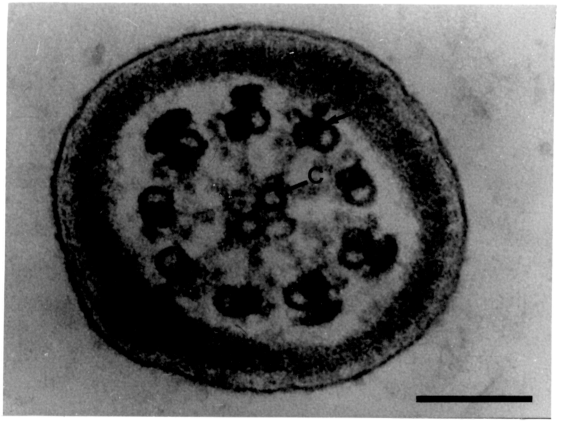


Figure A.9. Transverse section of principle piece of the tail of frozen-thawed sperms showing a fibrous sheath (arrow), nine axoneme doublets (D) and a central pair (C). Scale bar: 0.1 μm

APPENDIX 3

ANNUAL GENERAL MEETING

and

THIRD SCIENTIFIC CONFERENCE

of the

ELECTRON MICROSCOPY SOCIETY MALAYSIA

PROCEEDINGS

November 19 - 21, 1993

Novotel, Penang

FINE STRUCTURE OF GOAT OOCYTES MATURED IN VITRO

Yusoff, M.*, Rajikin, M.H., Ragunathan, T.
and Abdullah, R.B.
Institute of Advanced Studies and Department of Zoology,
University of Malaya, 59100 Kuala Lumpur, Malaysia
and Department of Physiology, Medical Faculty,
Universiti Kebangsaan Malaysia, Jalan Raja Muda,
50300 Kuala Lumpur, Malaysia.

SUMMARY

The structure and distribution of organelles within different groups of goat oocytes at various stages of in vitro maturation (0 hr, 20 hrs and 40 hrs) were studied. Oocytes were classified into Groups 1, 2 and 3, corresponding to oocytes with five or more layers of cumulus cells, those with 2-4 layers and cumulus-free oocytes, respectively. Comparison between oocytes was done on the basis of the development, impregnation and zonation of their zona pellucida, the structural appearance and distribution of mitochondria, cortical granules, endoplasmic reticula, ribosomes, lipid bodies, vesicles as well as clusters of membrane-bound electron-transparent bodies. These ultrastructural observations were correlated with their physiological functions and used to predict the attainment of full maturation for the purpose of in vitro fertilization.

INTRODUCTION

Mammalian primary oocytes reach the diplotene stage of meiosis at approximately the time of birth (Brambell, 1956; Zuckermann, 1960). The germinal material of the oocytes is then arranged within the vesicular nucleus, the dictyate stage, after which it remains arrested until a few hours prior to ovulation. Chang (1955) demonstrated that primary oocytes are capable of resuming meiotic maturation spontaneously following their removal from the respective follicles and cultured in vitro. In vitro maturation of goat oocytes has been studied (De Smedt et al., 1992; Hanada, 1985; Kim et al., 1984; Song and Iritani, 1985; Younis et al., 1991) although not on the basis of their ultrastructure. Fleming and Saake (1972) and Kruip et al. (1983) described the fine structure of matured oocytes of cattle while Cran et al. (1980) studied those of sheep. However, these observations were of oocytes matured in vivo.

The present study is aimed at describing the ultrastructural changes during in vitro maturation of different groups of goat oocytes.

MATERIALS AND METHODS

Collection and washing of oocytes were carried out following the procedure described by Rajikin et al. (unpublished). Oocytes were classified as those surrounded by five or more layers of cumulus cells (Group 1), those with 2-4 layers (Group 2) and cumulus-free oocytes (Group 3).

Collected and washed oocytes were incubated in a modified in vitro maturation medium (IVM-TCM 199) described by Younis et al. (1991). TEM studies were done on oocytes from each Group after 0, 20 and 40 hours of incubation.

Agar-embedded oocytes were prefixed in 3% glutaraldehyde with 0.5% formaldehyde in 0.1M phosphate buffer (pH7.4), postfixed in 1% osmium tetroxide in 0.1M cacodylate buffer (pH7.4) and block stained with 2% uranyl acetate in 0.005% acetic acid. Standard TEM preparation procedures followed and then viewed at 80kV.

RESULTS AND DISCUSSION

Group 1 oocytes

At 0 hr of maturation, zona pellucida had developed although zonation was not evident. Lipid bodies were present but no mitochondria were observed. At 20 hrs, zona pellucida had differentiated into thicker inner and thinner outer regions. Clusters of membrane-bound electron-transparent bodies were present in the perivitelline space. Mitochondria were fully developed, distributed evenly and usually in close proximity with dilated endoplasmic reticula, while cortical granules were generally at the periphery (Fig. 1). A number of mitochondria were hooded at 40 hrs of maturation.

Group 2 oocytes

In immature (0 hr) oocytes, zona pellucida was penetrated by cumulus cell processes and the mitochondria were not well developed. However, in matured (20 hrs) oocytes, fully developed mitochondria, many of which were hooded (Figs 2,3), could be observed. Clusters of membrane-bound electron-transparent bodies were present while cortical granules were distri-buted at the periphery (Fig. 2).

Group 3 oocytes

Zonation within the zona pellucida of immature (0 hr) oocytes was indistinct. Very few vesicles and lipid bodies were observed. Mitochondria in mature (20 and 40 hrs)

oocytes were sparsely distributed, not well developed and lacked cristae (Fig. 4). At 40 hrs, zona pellucida was less compact and membrane-bound electron-transparent bodies were less numerous compared to those of other Groups. Endoplasmic reticula were not dilated and cortical granules were few with no definite pattern of distribution.

No maturation, or a low maturation rate, of bovine oocytes occurred when cumulus cells were removed (Dalhausen et al., 1981; Leibfried and First, 1976). Critser et al. (1986) reported a higher rate of embryonic development following in vitro fertilization (IVF) of bovine cumulus-oocyte-complex (COC) matured in vitro compared to those of naked ones. Generally, the observations reported here are consistent with previous reports. The organelles appeared to be well developed in COC compared to those in naked ones. These ultrastructural observations also suggested that goat oocytes, particularly COC, had attained full maturation at 20 hrs to allow IVF procedures to be instituted.

REFERENCES

Brambell, F.W.R. 1956. Development of ovary and oogenesis. In: Marshall's Physiology of Reproduction, Third Edition Volume 1 (Ed. Parker, A.S.), Longman, London.

Chang, M.C. 1955. The maturation of rabbit oocytes in culture and their maturation, activitation, fertilization and subsequent development in the fallopian tubes. J. Exp. Zool., 128:379-405.

Cran, D.G., Moor, R.M. and Hay, M.F. 1980. Fine structure of the sheep oocyte during antral follicle development. J. Reprod. Fert., 59:125-132.

Critser, E.S., Leibfried-Rutledge, M.L., Eyestone, W.H., Northey, D.L. and First, N.L. 1986. Acquisition of developmental competence during maturation in vitro (abstract). Theriogenology, 25:150.

Dalhausen, R.E., Bonham, J.B., Meyers, G. and Ludwick, G. 1981. Characterization and maturation of prepuberal calf follicular oocytes in vitro (abstract). Theriogenology, 15:111.

De Smedt, V., Crozet, N., Ahmed-Ali, M., Martino, A. and Cognie, Y. 1992. In vitro maturation and fertilization of goat oocytes. Theriogenology, 37:1049-1060.

Fleming, W.N. and Saake, R.G. 1972. Fine structure of the bovine oocyte from the mature Graafian follicle. J. Reprod. Fert., 29:203-213.

Hanada, A. 1985. In vitro fertilization in goat. Jpn. J. Anim. Reprod., 31:21-26.

Kim, C.I., Imai, H., Niwa, A.K. and Iritani, A. 1984. Maturation division of goat follicular oocytes cultured in vitro. Korean J. Anim. Sci., 26:435-439.

Kruip, T.A.M., Cran, D.G., Van Beneden, T.H. and Dieleman, S.J. 1983. Structural changes in bovine oocytes during final maturation in vivo. Gam. Res., 8:29-47.

Leibfried, M.L. and First, N.L. 1976. Characterization of bovine follicular oocytes and their ability to mature in vitro. J. Anim. Sci., 48:76-86.

Song, H.B. and Iritani, A. 1985. In vitro fertilization of goat follicular oocytes with epididymal spermatozoa capacitated in a chemically defined medium. Proc. 3rd AAAP. Anim. Sci. Congr. Seoul, Korea, Volume 1, pp. 463-465.

Younis, A.I., Zuelke, K.A., Harper, K.M., Oliveira, M.A.L. and Brackett, B.G. 1991. In vitro fertilization of goat oocytes. Biol. Reprod., 44:1177-1182.

Zuckermann, S. 1960. Origin and development of oocytes in foetal and mature mammals. Mem. Soc. Endocr., 7:63.

The effects of freezing on the ultrastructure of goat sperm

M.H. RAJIKIN[1], M. YUSOFF[2], and R.B. ABDULLAH[3]
[1]Department of Physiology, Faculty of Medicine,
Universiti Kebangsaan Malaysia, Jalan Raja Muda, 50300 Kuala Lumpur, Malaysia.
[2]Institute of Advanced Studies, Universiti Malaya, 59100 Kuala Lumpur, Malaysia.
[3]Department of Zoology, Faculty of Science, Universiti Malaya, 59100 Kuala Lumpur, Malaysia.

ABSTRACT

The ultrastructure of frozen-thawed goat sperm was compared with those of freshly ejaculated sperm. Comparison was done in terms of the morphology of the head, the presence or absence as well as substructure of the acrosome, the length and internal structure of the neck and different regions of the tail. The study has revealed no apparent differences in ultrastructure between the frozen-thawed and the freshly ejaculated sperm.

TRODUCTION

The success for the routine *in vitro* fertilisation-bryo transfer (IVF-ET) and artificial insemination I) procedures very much depend upon the continuous pply of sperm. One of the ways this could be ieved is by freezing the freshly ejaculated sperm in uid nitrogen until needed. Although the use of goat zen sperm for the ET and AI procedures has revealed ying degree of success, information regarding the rastructure of this sperm is not available. This ormation is important to ascertain whether freezing any effect on the ultramorphology of the sperm ce this could affect their fertilisability. The purpose this study was to compare the ultrastructure of zen-thawed goat sperm with those of freshly culated one.

ATERIALS AND METHODS

Male Katjang goats maintained and reared at the n of the Institute of Advanced Studies of the versity of Malaya were used to collect fresh sperm. nale Katjang goats which were on natural oestrus re used as teasers. Semen was collected from male t using artificial vagina (AV). In the laboratory the rmatozoa were washed with washing medium. zen semen was obtained from Animal Reproductive oratory, Institute of Advanced Studies, University Malaya. After thawing in a water bath at 37°C, rmatozoa were washed as before for the freshly culated sperm. Both samples were subjected to smission electron microscopy (TEM) for morphological analyses. In this procedure the sperm were initially centrifuged prior to fixation. The pellet was primarily fixed in 3% (vv) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4), post fixed in 1% (w/v) osmium tetroxide in 0.1 M sodium cacodylate buffer (pH 7.4) and stained with 2% uranyl acetate in 0.005% acetic acid for 15 minutes at room temperature. Dehydration in a graded ethanol series was followed by two changes in propylene oxide and embedded in Mollenhauer's Epon-araldite resin (3). Ultrathin sections were stained with uranyl acetate and lead citrate prior to examination in a Philips CM12 transmission electron microscope operated at 80 kV.

RESULTS

Sperm head

The head of both freshly ejaculated and frozen-thawed sperm was elongated and consisted of an electron-dense nucleus with a pointed tip (Figures 1, 2 and 3), a less electron-dense acrosome, bounded by an inner and outer acrosomal membranes, with a bulbous tip (Figures 1 and 2) and extended down two-thirds of the length of the nucleus (Figures 2 and 3). The basal one-third of the nucleus was loosely surrounded by an electron-dense postacrosomal sheath (Figures 2 and 3) while the whole head, 6.5-8.5 μm long, was enveloped by an undulating plasma membrane (Figure 1), although it was frequently not observed in frozen-thawed sperm (Figures 2 and 3). Occasionally, the frozen-thawed sperm was observed to lack any acrosomal material (Figure 3).

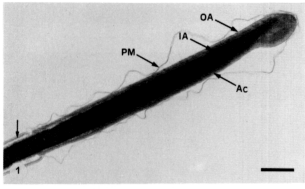


Figure 1. Longitudinal section (LS) of part of freshly-ejaculated of the head showing undulating plasma membrane (PM), inner (IA) and outer acrosomal membranes (OA), acrosome (Ac) with a bulbous tip, nucleus (N) and postacrosomal sheath (arrow). Scale bar : 0.5 μm

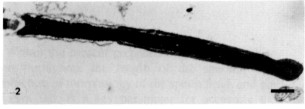


Figure 2. LS of the frozen-thawed the head showing the absence of plasma membrane. Scale bar : 0.5 μm

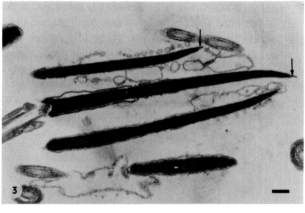


Figure 3. LS of several of the frozen-thawed heads showing the absence of plasma membrane and acrosomal contents. Note the presence of subacrosomal space (arrow). Scale bar : 0.5 μm

Sperm tail

In both freshly ejaculated and frozen-thawed sperm, the tail had a mid-piece (Figures 4 and 5), 0.6-1.2 μm in diameter, which consisted of tail fibres and microtubules, surrounded by a mitochondria sheath, and a principal piece (Figure 6), 0.2-0.6 μm in diameter, which comprised a similar arrangement of fibres and microtubules but the mitochondria sheath was replaced by a fibrous sheath. Throughout the length of the tail, the microtubules consisted of nine axoneme doublets arranged in a ring with a central pair. Each axoneme doublet in the mid-piece has a corresponding electron-dense outer fibre which lie towards the inside of the mitochondria.

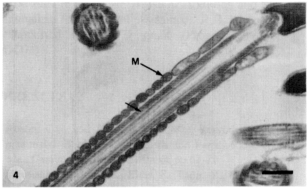


Figure 4. LS of part of freshly-ejaculated of the mid-piece showing mitochondrial sheath (M) and tail fibres/microtubules (arrow). Scale bar : 0.5 μm

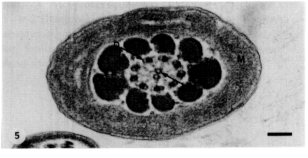
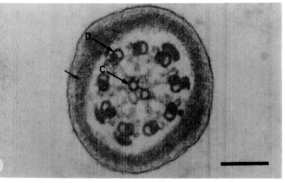


Figure 5. Transverse sections of freshly-ejaculated of the mid-piece showing mitochondrial sheath (M), 9 outer dense fibres (F), 9 axoneme doublets (D) and central pair (C). Scale bar : 0.1 μm



ure 6. Transverse sections of frozen-thawed ciple piece showing fibrous sheath (arrow) nine er dense fibres (F), nine axoneme doublets (D) and tral pair (C). Scale bar : 0.1 μm

CUSSION

Within the present preparation, it was difficult to ion throughout the length of the sperm. The astructure of morphology of the sperm head, and not sperm tail, is a key parameter for accessing the m fertilising competence *in vitro* (2). The present y has shown that the head of both freshly ejaculated frozen-thawed sperm was elongated. The gated head of the frozen-thawed bovine sperm has been reported (4). Regarding sperm acrosome, tin and Short (1) noted that the exact shape of the some is species dependent. Up to this date, as far e are aware, there was no similar study reported in . In the present study, we found that there was a dense acrosome, bounded by an inner and outer some membranes in both groups of sperm.

The main function of tail is for motility. The high city of metabolism of the mid-piece of the tail is ected by the presence of mitochondrial helix. The presence of microtubules consisted of nine axoneme doubles arranged in a ring with a central pair in both groups of sperm suggests that there was no impairment in the sliding movement of microtubules and bending motion of the sperm. In the bovine species, the frozen-thawed and freshly collected semen provided similar rates of penetration following IVF (5). Therefore, from our ultrastructural study of sperm morphology, it can be assumed that freezing does not impair the fertilisability of goat sperm.

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REFERENCES

(1) Austin, C.R. and Short, R.V. (1982). Reproduction in mammals. Book I: Germ Cells and Fertilisation, 2nd edn., Cambridge University Press. pp 63-71.

(2) Mashiach, R., Fisch, B., Eltes, F., Tadir, Y., Ovadia, J. and Bartoov, B. (1992). The relationship between sperm ultrastructural features and fertilising capacity *in vitro*. *Fertil. Steril.*, **57**: 1052-1057.

(3) Mollenhaeur, H.H. (1964). Plastic embedding mixtures for use in electron microscopy. *Stain Technology*, **39**: 111-114.

(4) Parrish, J.J., Susko-Parrish, J., Winer, M.A. and First, N.L. (1988). Capacitation of bovine sperm by heparin. *Biol. Reprod.*, **38**: 1171-1180.

(5) Pavlock, A., Torner, H., Motlik, J., Fulka, J., Kauffold, P. and Duschinski, U. (1988). Fertilisation of bovine oocytes *in vitro*: Effect of different sources of gametes on fertilisation rate and frequency of fertilisation anomalies. *Anim. Reprod. Sci.* **16**: 209-213.

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ULTRASTRUCTURAL STUDIES OF DEVELOPING GOAT OOCYTES IN VITRO

M.H. Rajikin,[1] M.Yusoff [2] and R.B. Abdullah [3]

[1] Department of Physiology, Medical Faculty
National University of Malaysia, 50300 Kuala Lumpur, Malaysia
[2] Institute of Advanced Studies, University of Malaya
59100 Kuala Lumpur, Malaysia,
[3] Department of Zoology, University of Malaya
59100 Kuala Lumpur, Malaysia

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ABSTRACT

The structure and distribution of organelles within developing goat oocytes at various stages of incubation were studied. In oocytes with 5 or more layers of cumulus cells, at 0 h of incubation, the zona pellucida had developed although zonation was not evident. Lipid bodies were present but no mitochondria were observed. At 20 h, the zona pellucida had differentiated into thicker and thinner regions. Clusters of membrane-bound electron-transparent bodies were present in the perivitelline space. The mitochondria were fully developed, distributed evenly and usually in close proximity with dilated endoplasmic reticula. Cortical granules were distributed at the periphery. At 40 h of incubation, a number of mitochondria was hooded. In oocytes of 2 to 4 layers of cumulus cells at 0 h, the zona pellucida was penetrated by cumulus cell processes, and the mitochondria were not well developed. However, in 20-h incubated oocytes, fully developed mitochondria, many of which were hooded, could be observed. Clusters of membrane-bound electron-transparent bodies were also observed, while cortical granules were at the periphery. In cumulus-free oocytes, zonation within the zona pellucida was indistinct. Very few vesicles and lipid bodies were observed. At 20 h, mitochondria were sparsely distributed and were not well developed and lacked cristae. At 40 h, the zona pellucida was less compact, and the membrane-bound electron-transparent bodies were less numerous compared with those of the other groups. Endoplasmic reticula were not dilated, and cortical granules were few and had no definite pattern of distribution.

Key words: goat, in vitro, oocyte development, ultrastructure

Acknowledgments

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INTRODUCTION

Mammalian primary oocytes reach the diplotene stag meiosis at approximately the time of birth (1,25). The ger material of the oocytes is then arranged within the vesi nucleus, the dictyate stage, after which it remains arr until few hours before ovulation. However, the primary oo are capable of resuming meiotic maturation spontaneously removed from their follicles and cultured in vitro Development of goat oocytes in vitro has been stu (11,9,20,24,7), but no fine structural basis for the evalu of such oocytes has been demonstrated. Among farm anima appears that pig (16), cattle (8,12) and sheep (3) were the animals in which the fine structure of developing oocyte ever been demonstrated.

It is the purpose of this paper to describe ultrastructural changes that occur in different groups of oocytes at various stages of development in vitro.

MATERIALS AND METHODS

Oocyte Collection

The ovaries in this study were obtained from an abatt They were rinsed and kept in 10 ml of washing medium (mH 199, hepes buffered H-TCM 199) (Sigma Chemical Co., cat. M2520 St. Louis, MO) with Earle's salt and L-glutamine, whic modified by addition of sodium pyruvate, 112 μg/ml streptomycine sulphate, 50 μg/ml at pH7.4) and transported wi 45 min to the laboratory. The surface of the ovaries was sl to release the oocytes. The ovaries were then flushed thoro with the washing medium. The collected oocytes were classifie follows: Group-1 oocytes were those with 5 or more layer cumulus cells, Group-2 were surrounded by 2 to 4 layer cumulus cells, and Group-3 were those of cumulus-free.

Care was taken to pick up only oocytes with a nor uncracked zona pellucida, having ooplasm with a granul appearance and which filled the entire perivitelline space. is important because some cumulus-free oocytes are from atr follicles which would not show developing characteristics in ultrastructure. Therefore, the cumulus-free oocytes wit cracked zona pellucida and partly filled perivitelline sp with smooth appearance of ooplasm were not chosen. Based on classification, a total of 90 oocytes, comprising 30 ooc from each group, were collected.

Incubation Medium and Oocyte Treatment

The medium used in this study was a modified TCM described by Younis et al. (24). The classified and was oocytes were transferred to 50-μL droplets of incubation me

(modified IVM-TCM 199). This medium consisted of buffered TCM 199 with Earle's salt and L-glutamine (b-TCM 199, Sigma Chemical Co., cat. no. M 4530, St Louis, MO), which was modified by addition of sodium pyruvate (50 μg/ml); glucose (5.5 mg/ml); streptomycine sulphate (50 μg/ml); bovine serum albumin; BSA (4 mg/ml); and heat-inactivated estrous goat serum (20% v/v). The oocytes were maintained in droplets of IVM medium under silicon oil (Aldrich Chemical Co. Inc., Milwaukee, WI) and placed in an in humidified 5% CO_2 in air at 38.5°C for 0, 20 and 40 hr. Each microdrop contained less than 10 oocytes. At the end of each incubation period, 5 oocytes were taken from each group for the ultrastructural studies. For Groups 1 and 2, at 20 and 40 hr, only oocytes with fully expanded cumulus cells were taken since these were the presumed developing oocytes.

Ultrastructural Studies

Five oocytes from each of the 3 groups were first embedded in 1% (w/v) ion agar at 0, 20 and 40 h for easy handling. Agar blocks were then prefixed in 3% (v/v) glutaraldehyde with 0.5% (w/v) formaldehyde in 0.1 M sodium phosphate buffer, pH 7.4, at room temperature for 40 min. After washing in buffer, the agar blocks were postfixed in 1% v/v osmium tetroxide in 0.1 M sodium cacodylate buffer, pH 7.4, at room temperature for 40 min. Then 2% (w/v) uranyl acetate in 0.005% acetic acid was used as an *en bloc* stain, i.e., by immersing the agar blocks in the stain for 40 min at room temperature. Washing with distilled water was followed by dehydration in a graded ethanol series, which was then replaced by acetone. The agar blocks were embedded in Epon-Araldite resin (15). Ultrathin sections for TEM were obtained using a Reichert Ultracut E ultramicrotome and stained with uranyl acetate and lead citrate, and the ultrastructure of the oocytes was examined under a Philips CM12 transmission electron microscope at 80 kV.

RESULTS

Group-1 Oocytes

At 0 h, the oocytes had fully developed zona pellucida 6.0 to 7.8 μm thick, although zonation within it was not yet evident (Figure 1). Penetration by cumulus cell processes occurred throughout the zona pellucida, and these processes, with diameters ranging from 120 to 240 nm, were also observed in the perivitelline space. The oolemma was indistinct, and numerous vesicles 0.25 to 1.20 μm in diameter were distributed throughout the cytoplasm. Lipid bodies, 1.7 to 2.8 μm in diameter were present, but no mitochondrion was observed.

At 20 h, the zona pellucida had differentiated into a thicker and less fibrous inner region, and a thinner and more fibrous outer region, with a total thickness of 1.5 to 15.0 μm. As in the 0-h oocytes, cumulus cell processes occurred

throughout the zona pellucida as well as in the perivitelline space (Figure 2). The perivitelline space, which was lined on the inside by the oolemma, was more extensive and its thickness varied greately (1.5 to 14.5 μm) from one region to another. Clusters of membrane-bound electron-transparent bodies occurred in some regions of the perivitelline space (Figure 2). Vesicles of 1.5 to 5.0 μm in diameter were present throughout the cytoplasm, although occurrence of fusion between adjacent vesicles could not be ascertained owing to membrane disintegration. Fully developed mitochondria (0.42 to 1.0 μm in diameter) were distributed evenly in the cytoplasm, usually in close proximity with dilated endoplasmic reticula, while electron-dense cortical granules had a peripheral distribution (Figures 2,3).

At a later stage of incubation (40 h) the zona pellucida appeared to be thinner (4.0 to 5.0 μm) but with similar zonation pattern and larger lipid bodies (2.5 to 7.0 μm in diameter) than those in the earlier stages. Most of the vesicles had disintegrated membranes. Junctions between the cumulus cell processes and the oolemma were observed (Figure 5). As at 20 h, the mitochondria had an even distribution within the cytoplasm, although they were always associated with endoplasmic reticula, while cortical granules were located mostly at the periphery (Figures 4,5,6). A number of mitochondria were hooded (Figure 6). Endoplasmic reticula were less dilated and some were in close proximity with the oolemma with no associated ribosomes. Membrane-bound electron-transparent bodies occurred in large clusters within the zona pellucida, with an electron-transparent region towards the outside of the clusters, but either singly or in groups of 2 or 3 throughout the cytoplasm (Figure 6).

Group-2 Oocytes

The zona pellucida of 0-h incubated oocytes had indistinct demarcation, with thickness ranging from 3.1 to 4.0 μm, and was deeply penetrated by cumulus cell processes (Figure 7). The oolemma was intact and formed microvilli. Numerous junctions between cumulus cell processes and the oolemma were observed, although extension into the cytoplasm was not common. As in Group 1, clusters of membrane-bound electron-transparent bodies (35.0 to 75.0 nm in diameter) occurred in clusters, with electron-transparent region around them, within the zona pellucida and in the narrow region between the oolemma and the zona pellucida, and either singly or in groups of 2 or 3 throughout the cytoplasm. Mitochondria were not well developed at this stage and were mostly distributed at the periphery. Cortical granules, some of which were clearly membrane-bound, occurred in close proximity with the oolemma. Numerous vesicles and some lipid bodies were observed, the former with disintegrated membranes.

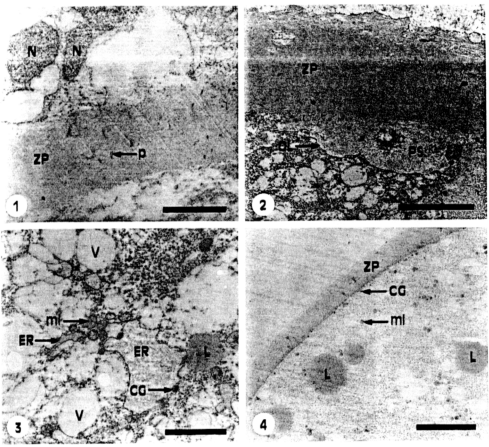

Figure 1. Group-1 oocytes incubated at 0 h. Part of transverse section (TS) showing the zona pellucida (ZP) penetrated by cumulus cell processes (p). N is the nucleus of the cumulus cell.

Figure 2. Group-1 oocytes incubated at 20 hours. Part of TS showing zonation within the zona pellucida and the perivitelline space (PS) lined on the inside by the oolemma (OL).

Figure 3. Higher magnification of part of section in Figure 2, showing mitochondria (mi), dilated endoplasmic reticulum (ER), vesicles with disintegrated membranes (V), cortical granules (CG) and lipid bodies (L).

Figure 4. Group-1 oocytes at 40 hours. Part of the TS showing evenly distributed mitochondria, some of which are hooded.

Scale bars: Figure 1 = 5 μm; Figures 2,4 = 10 μm; Figure 3 = 3 μm

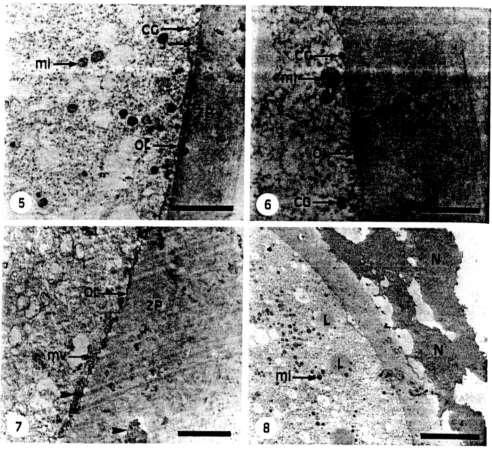

Figure 5. Group-1 oocytes incubated at 40 hours. Parts of transverse section (TS) showing peripheral distribution of cortical granules (CG) and even distribution of mitochondria (mi).

Figure 6. Note the close proximity of the cortical granules to the oolemma (OL), presence of junctions (J) between cumulus cell processes and oolemma, and clusters of membrane-bound bodies (arrowhead) within zona pellucida (ZP).

Figure 7. Group-2 oocytes incubated at 0 hr. Part of TS showing microvilli (mv) and clusters of membrane-bound bodies (arrowhead) within zona pellucida and the region between oolemma and zona pellucida.

Figure 8. Group 2 oocytes incubated at 20 hours. Part of TS showing cumulus cells, each with a nucleus (N), hooded mitochondria and lipid bodies (L).

Scale bars:Figure 5 = 4 μm; Figures 6 and 7 = 2 μm; Figure 8 = 10 μm.

Oocytes at 20 h had a similarly-zoned fibrillar zona pellucida 3.5 to 5.5 μm thick, with sparse cumulus cells on the outside forming processes which penetrated the former (Figure 8). There are fully developed mitochondria, a large proportion of which were hooded (Figure 9) could be observed throughout the cytoplasm, although peripheral distribution was evidently preferred. Clusters of membrane-bound electron-transparent bodies, with associated electron-transparent boundary, occurred in isolatedregions of the zona pellucida and the region between the oolemma and zona pellucida, causing invaginations of oolemma in the latter case (Figure 10). Similar bodies were also observed in smaller group throughout the cytoplasm, especially near the clusters of mitochondria. Lipid bodies were relatively large (2.1 to 4.6 μm in diameter), while the few vesicles present had disintegrated membranes. Most of the cortical granules were at the periphery, some were not membrane-bound and were associated closely with invaginations of the oolemma (Figure 10).

Group-3 Oocytes

In these oocytes cumulus cells were absent. At 0 h, zonation within the 5.7 to 6.8 μm thick zona pellucida was indistinct, although a gradual outward increase in its fibrillar nature was observed (Figure 11). Penetration of the zona pellucida was only by microvilli formed by folding of the oolemma. Numerous mitochondria (0.32 to 0.96 μm in diameter) occurred in groups and was associated with dilated endoplasmic reticula throughout the cytoplasm, usually away from the nucleus. No cristae were observed within the mitochondria (Figure 12). Endoplasmic reticula were attached to ribosomes and no free ribosomes were observed. The nuclear membrane had numerous pores and seemed contiguous with dilated endoplasmic reticula. Very few vesicles, with disintegrated membranes and lipid bodies, were observed.

Similar substructure and pattern of the zona pellucida, 3.0 to 3.8 μm thick were observed at 20 h. The oolemma formed microvilli, although no penetration of the zona pellucida was observed. Mitochondria were relatively sparse (Figure 13) and not well developed (0.34 to 1.4 μm in diameter), and fewer cristae were observed (Figure 14). As in all other oocytes described previously, membrane-bound electron-transparent bodies occurred in large clusters, with electron-transparent periphery, within the zona pellucida (Figure 14) and in smaller groups throughout the cytoplasm. Dilated endoplasmic reticula were not prominent and free ribosomes were absent. Very few relatively large vesicles, with disintegrated membranes, and membrane-bound cortical granules were observed particularly at the periphery.

The zona pellucida at 40 h was 3.2 to 4.1 μm thick and it appeared to be less compact towards the outside, although it was fibrillar throughout (Figure 15). Similar clusters of membrane-bound electron-transparent bodies occurred in the zona

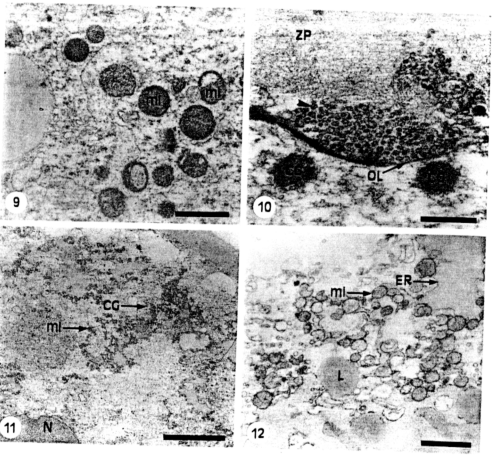

Figure 9. Group-2 oocytes incubated at 20 hours. Sections showing hooded mitochondria (mi.)

Figure 10. Membrane-bound bodies (arrowhead) enclosed between the invaginated oolemma (OL) and the fibrous zona pellucida (ZP) with associated non-membrane-bound cortical granules (CG).

Figure 11. Group-3 oocytes incubated at 0 hr. The transverse section (TS) showing distribution of mitochondria and cortical granules. N is the nucleus.

Figure 12. Higher magnification of part of section in Figure 11 showing association of mitochondria and dilated endoplasmic reticula (ER).

Scale bars: Figure 9 = 1 μm; Figure 10 = 0.3 μm; Figure 11 = 15 μm; Figure 12 = 2 μm.

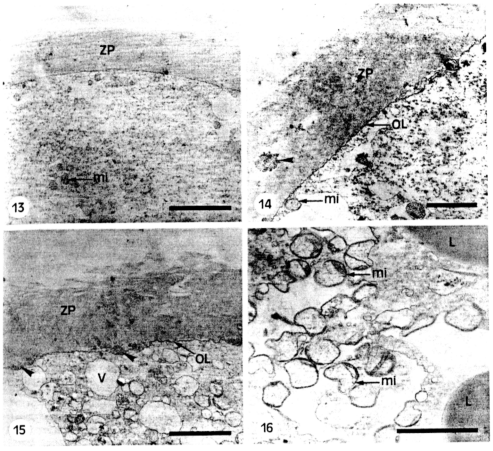

Figure 13. Group-3 oocytes incubated at 20 hours. Parts of transverse section (TS) showing the scarcity of organelles within the cytoplasm.

Figure 14. Note the presence of the cluster of membrane-bound bodies (arrowhead) within the zona pellucida (ZP).

Figure 15. Group-3 oocytes incubated at 40 hours. Parts of TS showing clusters of membrane-bound bodies (arrowhead) enclosed between the invaginated oolemma (OL) and zona pellucida.

Figure 16. Abnormally-shaped mitochondria (mi) with few or no cristae.

Scale bars: Figure 13 = 5 μm; Figures 14 and 16 = 2 μm; Figure 15 = 3 μm.

pellucida and the region between the oolemma and zona pellucida, both more numerous compared with the oocytes described previously. These bodies were also observed throughout the cytoplasm in smaller clusters. Oolemma appeared intact and formed microvilli, although they were not very prominent. Mitochondria (0.42 to 1.2 μm in diameter) were small in number, irregular in shape, and had few or no cristae (Figures 15,16). Numerous vesicles of various sizes (1.0 to 8.0 μm in diameter) with intact membranes and few lipid bodies were present. Endoplasmic reticula were not dilated as in other groups of oocytes and with no associated ribosomes. Very few cortical granules with no definite pattern of distribution were observed.

DISCUSSION

The purpose of this study was to characterize the ultrastructure the cytoplasm of developing goat oocytes in vitro. Cumulus cell expansion was used as a criterion to indicate development process. It was not the intention of this study to describe the maturity of the oocytes, since the classic determination for this stage of development is based on observing extrusion of the second polar body.

The role of cumulus cells in the acquisition of full developmental competence of oocytes has been investigated (17,13,23). Leibfried and First (13) and Dalhausen et al. (5) showed that there was no maturation, or a low rate of maturation rate of bovine oocytes when the cumulus cells were removed before the oocytes were matured in vitro. Critser et al. (4) found a higher rate of embryonic development following in vitro fertilization (IVF) and culture of bovine cumulus-oocyte-complex (COC) matured in vitro compared with that of nude or corona-enclosed oocytes. Similar development patterns of cytoplasmic organelles were observed in the present study, indicating that these oocytes were undergoing normal maturing processes.

In all 3 groups, the zona pellucida had fully developed, but zonation within it was not evident. In COC (Groups 1 and 2), there were penetrations by cumulus cell processes, and this could be the route for the uptake of nutrients through the plasma membrane, which is needed for the oocyte development process (3). In Group-3 oocytes, however, such penetration was only by microvilli, probably due to the folding of the oolemma, and this structure does not seems to be changed even after 40 h of incubation. Cran et al. (3) in their studies on sheep oocytes did not seem to investigate such oocytes. However, their finding, that slender villi were only observed in oocytes of very small follicles, after which they shortened and became thicker, suggests that these oocytes were developing, unlike our present cumulus-free oocytes.

Localization of mitochondria at the periphery of the oocyte has been observed in cattle (8) and sheep (3). Our finding in goats showed that these mitochondria were usually in close proximity with endoplasmic reticula; however, in cattle (8) a dispersal mitochondria was associated with the formation of the perivitelline space. In sheep, although no association has been noted, the observation on the tight peripheral band in the earlier stage of development has also been reported (3). The hooded appearance of mitochondria in Groups-1 and 2, but not in Group-3 oocytes suggests the superiority of the COC in regard to the development of the oocytes. Furthermore, in Group-3 oocytes, there were relatively fewer, not well-developed mitochondria and there were fewer cristae compared to COC. Hooded appearance of mitochondria during late estrus has been reported in sheep (3) and in cattle (6,18). Hooding of mitochondria increased their surface area and may provide a specific micro-environment to facilitate exchange of metabolic intermediates with the endoplasmic reticulum (3).

Our finding also showed that the endoplasmic reticula were dilated. While in goats some of these endoplasmic reticula were in close proximity with the oolemma with no associated ribosome, in sheep they were related to the presence of extended surface villi (3). We are of the opinion that it is the oolemma which made the extended surface villi. It is assumed that surface villi represent a temporary storage of products produced during the rapid growth phase of the oocytes.

Cortical granules in COC at 0 h were numerous but dispersed randomly. At later stages of incubation (20 and 40 h), they were not only numerous but also distributed at the periphery, just under the plasma membrane. In cumulus-free oocytes there were very few cortical granules, with no definite pattern of distribution observed. This finding is similar to that in sheep that cortical granules were randomly scattered in small follicles but in the region of plasma membrane in the larger; whereas, in late estrus, these granules were localized immediately beneath the plasma membrane (3). It appears then that the late stage of cortical granule development found in goats is similar to that of sheep oocytes in late estrus. Szollosi et al. (21) in their studies on the rabbit suggested that this is a result of the loss of gap junctions between the foot of cumulus processes and the oocyte surface. Cran et al. (3) found the reduction in the relationship between the surrounding granulosa cells and the oocytes the result of degeneration of cumulus cells. However, different from that of sheep and rabbits, the present study showed that the gapjunction between the foot of cumulus processes and the oocyte surface remain intact. The peripheral localization of cortical granules in developing oocytes have some bearing on their subsequent fertilization. As has been shown in hamster oocytes, following the fusion of spermatozoa and oocytes, cortical granules reaction occurred whereby the oocyte plasma membrane fused with the membrane of the cortical granules,

releasing their enzymes into the perivitelline space and then seeping into the zona pellucida (22). These enzymes alter the zona pellucida glycoprotein constituent, rendering it inactive as a sperm receptor (22). Therefore, it is tempting to suggest that the number and the distribution of these organelles play a major role in the development process.

In all 3 groups of oocytes, regardless of their stages of development, clusters of membrane-bound electron-transparent bodies with associated electron-transparent boundary occurred in isolated regions of the zona pellucida and the region between the oolemma and zona pellucida, causing invagination of oolemma in the later case (Figure 10). Cran et al. (3) described similar structures in sheep as aggregation of particles or vesicles. However, these aggregations appeared to be larger in size. Kruip et al. (12) and De Loose et al. (6) did not report such structures in bovine oocytes. Matthews and Martin (14) had demonstrated the presence of annulate lamellae in human oocytes, which consists of double membranes with periodic annuli occurring in regular order. Such lamellae were not observed in our study of goat oocytes. However, the membrane-bound electron-transparent bodies appeared to have similar structures to the periodic annuli reported by Matthew and Martin (14). The physiological significance of these bodies has yet to be determined, particularly with regard to the development process, since they were also found in the cumulus-free oocytes.

The results of this study showed that Group-1 and 2 oocytes had undergone similar changes in their ultrastructures during development. In both groups there were also no notable differences in the ultrastructure of cytoplasmic organelles between 20 and 40 h of incubation. These observations probably not only suggest the superiority of Group-1 and 2 oocytes compared with those of Group 3, but also indicate that 20 h incubation was sufficient for the attainment of full development of oocytes. This possibility was reported by Shamsuddin et al. (19) who found that an incubation period of 20 to 24 h is sufficient for in vitro maturation of bovine oocytes. Although the present study shows no difference in the ultrastructure of cytoplasmic organelles between 20 and 40 h, it has been reported that bovine oocytes incubated beyond 26 h resulted in a reduced maturation rate (19), probably due to an increase in overmatured oocytes. Hyttel et al. (10) in their study on the ultrastructure of in vitro maturation of oocytes in cattle have also reported that such oocytes begin to degenerate 30 h after incubation. These findings warrant further study of nuclear changes and subsequent in vitro fertilization of developing goat oocytes cultured in vitro.

REFERENCES

1. Brambell FWR. Development of ovary and oogenesis. In Parkere AS (ed), Marshall's Physiology of Reproduction, Vol. 1, Longman, London, 1956; Chapter 5.

2. Chang MC. The maturation of rabbit oocytes in cultur and their maturation, activation, fertilization a subse quent development in the fallopian tubes. J Exp Zoo 1955;128:379-405.

3. Cran D, Moor RM, Hay M. Fine structure of the sheep oocyt during antral follicle development. J Reprod Fertil 1980;59:125-132.

4. Critser ES, Leibfried-Rutledge ML, Eyestone WH, Northey DI First NL. Acquisition of developmental competence during maturation in vitro. Theriogenology 1986;25:150 abstr.

5. Dalhausen RE, Bonham JB, Meyers G, Ludwick G Characterization and maturation of prepuberal calf follicula oocytes in vitro. Theriogenology 1981;15:111 abstr.

6. De Loose F, van Vliet C, van Maurik P, Kruip AM. Morpholog of immature bovine oocytes. Gam Res 1989;24:197-204.

7. De Smedt V, Crozet N, Ahmed-Ali M, Martino A, Cognie Y. I vitro maturation and fertilization of goat oocytes Theriogenology 1992;37:1049-1060.

8. Fleming WN, Saake RG. Fine structure of the bovine oocyt from the mature Graafian follicle. J Reprod Fertil 1972;29:203-213.

9. Hanada A. In vitro fertilization in goat. Jpn J Anim Repro 1985;31:21-26.

10. Hyttel P, Xu KP, Smith S, Greve T. Ultrastructure of i vitro oocyte maturation in cattle. J Reprod Fertil 1986;78:615-625.

11. Kim C. I., Imai H, Niwa AK, Iritani A. Maturation divisio of goat follicular oocytes cultured in vitro. Korean J Ani Sci 1984;26:435-439.

12. Kruip TAM, Cran DG, Van Beneden TH, Dieleman SJ. Structu al changes in bovine oocytes during final maturation in vivo Gam Res 1983;8:29-47.

13. Leibfried ML, First NL. Characterization of bovin follicular oocytes and their ability to mature in vitro. J Anim Sci 1976;48:76-86.

14. Matthews JL, Martin JH. Atlas of Human Histology and Ultra structure. Lea & Febiger, Philadelphia, 1971;204-217.

15. Mollenhauer HH. Plastic embedding mixtures for use in electron microscopy. Stain Technology 1964;39:111-114.

16. Norberg HS. Ultrastructure of pig tubal ova: Th unfertilized and pronuclear stage. Zellforsch 1973;141: 103-122.

17. Sato E, Iritani A, Nishikawa Y. Factors involved maturation of pig and cattle follicular oocytes cultured vitro. Jpn J Anim Reprod 1977;23:12-18.

18. Senger PL, Saake RG. Unique mitochondria in the bovi oocyte. J Cell Biol 1970;46:405-408.

19. Shamsuddin M, Larsson B, Rodriguez-Martinez Maturation-related changes in bovine oocytes under differe culture conditions. Anim Reprod Sci 1993;31:49-60.

20. Song HB, Iritani A. In vitro fertilization of goat follicul oocytes with epididymal spermatozoa capacitated in chemically defined medium. Proc 3rd AAAP Anim Sci Congr, Vo 1, Seoul, Korea, 1985; pp 463-465.

21. Szollosi D, Gerard Y, Menezo Y, Thibault C. Permeability ovarian follicle; corona cell-oocyte relationship in mammal Annls Biol Anim Biochem Biophys 1978;18:511-521

22. Wassarman PM. Fertilization in mammals. Sci Amer 198 Dec:52-58.

23. Xu KP, Greve T, Smith S, Hyttel P. Chronological changes bovine follicular oocytes maturation in vitro. Acta Vet Scan 1986;27:505-519.

24. Younis AI, Zuelke KA, Harper KM, Oliveira MAL, Bracket BG. In vitro fertilization of goat oocytes. Biol Repro 1991;44:1177-1182.

25. Zuckermann S. Origin and development of oocytes in foeta and mature mammals. Mem Soc Endocrinol 1960; 7:63 abstr.