IN VITRO STUDIES OF MATURATION AND FERTILIZATION OF OOCYTES AND SUBSEQUENT CULTURE OF EMBRYOS IN GOATS

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ABSTRACT

Information on in vitro maturation (IVM) and fertilization (IVF) of oocytes and subsequent culture (IVC) of embryos in goat worldwide is lacking. This study is an attempt to develop some procedures in a local set up for the maturation and fertilization of goat oocytes in vitro and to develop further the embryos thus obtained into postembryonic stage. The study involved the use of oocytes from ovaries of dead animals taken immediately after slaughter. For this purpose, the collected oocytes were subjected to maturation processes, and their developmental competence was studied based on the cytoplasmic changes by means of light and electron microscopy, and chromosomal changes. Oocytes which attained presumable maturity were inseminated with heparin-treated frozen-thawed sperm for fertilization.

The goat oocyte was spherical in shape, 110 to 120 μm in diameter and consisted of a spherical mass of cytoplasm which was surrounded by thick transparent membrane, zona pellucida. At 0 hr of incubation in the in vitro maturation medium (tissue culture medium, TCM 199), the cumulus (cu) and corona cells (co) of cumulus-oocyte complex (COCs) were tightly packed. Lipid bodies were present but no mitochondria were observed. The zona pellucida had attained full development but the zonation was not evident. There were penetration by cumulus cell processes into the zona pellucida and into perivitelline space. At 14 hr, the cumulus

cells begin to expand and loosen, however the corona cells remained tightly packed. At 20 hr, both cumulus and corona cells reached maximum expansion and loosening, and did not change any further even after 40 hr of incubation. The mitochondria, a number of which were hooded were fully developed, distributed evenly and usually in close proximity with dilated endoplasmic reticula. Cortical granules were distributed at the periphery. The zona pellucida had differentiated into thicker and thinner regions. At 40 hr, the expansion and loosening of the cumulus cells and the corona cells was not of any difference from those of 20 hr. A number of mitochondria were hooded. The zona pellucida appeared thinner but with similar zonation pattern and larger lipid bodies compared to those in the earlier stages.

In cumulus-free oocytes (CFOs), the cumulus and corona cells were absent. At 0 hr, very few vesicles and lipid bodies were observed. At 20 hr the mitochondria were sparsely distributed, not well developed and lacked cristae. The cortical granules were few and had no definite pattern of distribution. These findings suggested that COCs were presumably more superior compared to CFOs in terms of their subsequent viability and fertilizability. In COCs, since there was no notable difference in the ultrastructure of cytoplasmic organelles between 20 and 40 hr of incubation, it is possible that 20 hr incubation was sufficient for such oocytes to attain fertilizable stage.

Such possibility was further investigated by looking at the chromosomal configuration of oocytes during culture in vitro. At 0 hr of incubation, most of the oocytes were at prophase 1, particularly at pachytene stage. At 20 to 30 hr, the period whereby the cytoplasmic changes were examined, more than 55% (43/78) of the COCs attained metaphase II (MII), whereas for CFOs the majority of these oocytes (40%, 8/20) remained at germinal vesicle breakdown (GVBD) stage and only 20% (4/20) of them reached MII. These findings supported the data on cytoplasmic changes that the COCs, and not CFOs, incubated between 20 to 30 hr were those which will presumably produce higher fertilization rate in the subsequent IVF procedure. For this reason, only COCs that were incubated for 20 hr will be used for the proceeding IVF study.

The present IVF studies on COCs, inseminated with frozen-thawed sperm in the fertilization medium supplemented with heparin and co-cultured with oviductal epithelial cells have shown that 12.5% (7/56) of the oocytes reached a 2-cell embryo stage. Further culture of these embryos have resulted on further development in 5.3% (3/56) of them into 4-cell stage. Since there was no further pre-embryonic development beyond 4-cell stage, it appears that there was a developmental block between 4- and 8-cell stage.

The present study has further demonstrated that the failure of oocytes co-cultured with epithelial cells but not inseminated to cleave suggests that the embryos obtained through the present system were unlikely as a result of parthenogenesis. The study also indicated that the failure of the inseminated oocytes to fertilize in an epithelial cells-free system suggests that the possible growth factor(s) secreted by the oviductal epithelial cells was/were necessary for fertilization and/or development.

In conclusion, the studies showed that using proper media and conditions, oocytes from ovaries of recently dead animals is possible to be matured and fertilized in vitro, and the embryos thus obtained could be subsequently cultured for preimplantation embryonic development. However, this may need the basic requirement including the capacitation of sperm with heparin and oviductal epithelial cells as co-culture for IVF and IVC.

ABSTRAK

Maklumat mengenai pematangan (IVM) dan persenyawaan (IVF) oosit in vitro dan seterusnya pengkulturan (IVC) embrio pada kambing di seluruh dunia sangat kurang. Kajian ini merupakan satu usaha membentuk suatu kaedah pematangan dan persenyawaan oosit kambing in vitro dan seterusnya pengkulturan embrio yang diperolehi melalui persenyawaan tersebut. Kajian ini melibatkan penggunaan oosit yang diambil dari haiwan yang baru disembelih. Bagi maksud ini, oosit yang dikumpulkan dimatangkan, dan kebolehannya berkembang dikaji berdasarkan perubahan sitoplasma, melalui mikroskopi cahaya dan mikroskopi elektron, dan perubahan konfigurasi kromosom. Oosit yang berkemungkinan mencapai kematangan diinseminasi dengan sperma sejuk-beku yang telah dirawat dengan heparin.

Oosit kambing berbentuk sfera, berdiameter 110-120 μm dan terdiri daripada sitoplasma yang juga berbentuk sfera, dan dikelilingi oleh membran lutsinar yang tebal, zona pelusida. Pada pengeraman 0 jam di dalam medium pematangan in vitro (medium kultur tisu, TCM 199), sel-sel kumulus (cu) dan korona (co) kompleks kumulus-oosit (COC) tersusun secara rapat. Terdapat jasad lipid tetapi mitokondria tidak terlihat. Zona pelusida mencapai perkembangan yang penuh tetapi zonasi tidak terlihat. Terdapat penembusan oleh proses-proses sel kumulus ke dalam zona pelusida dan ke dalam ruang perivitelin. Pada 14 jam, sel-sel kumulus mula mengembang dan longgar, walaubagaimanapun sel-sel korona tetap tersusun

rapat. Pada 20 jam, kedua-dua sel kumulus dan sel korona mencapai pengembangan dan pelonggaran yang maksimum, dan tidak mengalami pengembangan selanjutnya walaupun selepas dieram selama 40 jam. Mitokondria, yang sebahagiannya bercangkuk, berkembang dengan sempurna, tersebar dengan merata dan selalunya berdekatan dengan retikulum endoplasma. Bintil-bintil korteks tersebar di periferi. Zona pelusida berdiferensiasi kepada bahagian tebal dan tipis. Pada 40 jam, pengembangan dan pelonggaran sel-sel kumulus dan korona tidak berbeza dengan keadaan pada 20 jam. Terdapat sebilangan mitokondria yang bercangkuk. Zona pelusida kelihatan lebih nipis tetapi mempunyai bentuk zonasi yang serupa dan jasad lipid yang lebih besar berbanding dengan keadaan pada peringkat lebih awal.

CFO tidak mempunyai sel-sel kumulus dan korona. Pada 0 jam, sejumlah kecil vesikel dan jasad lipid terlihat. Pada 20 jam mitokondria kurang tersebar, tidak berkembang dengan baik dan ketiadaan kristae. Jumlah bintil-bintil korteks hanya sedikit sahaja dan tidak mempunyai bentuk taburan yang tertentu. Penemuan in mencadangkan bahawa COC mungkin lebih superior berbanding dengan CFO daripada segi kebolehtahanan dan kebolehsenyawaannya. Pada COC, oleh kerana tidak terdapat perbezaan ultrastruktur pada organel-organel sitoplasma di antara waktu pengeraman 20 dan 40 jam, adalah mungkin bahawa pengeraman 20 jam mencukupi untuk oosit tersebut mencapai peringkat kebolehsenyawaan.

Kemungkinan di atas dikaji selanjutnya dengan cara melihat konfigurasi kromosom oosit semasa kultur in vitro. Pada pengeraman 0 jam, kebanyakan oosit berada pada peringkat profasa 1, terutamanya peringkat pakiten. Pada 20 hingga 30 jam, iaitu waktu di mana perubahan sitoplasma diperiksa, lebih daripada 55% (43/78) COC mencapai metafasa II (MII), sementara untuk CFO majoriti oosit ini (40%, 8/20) tetap pada peringkat pemecahan vesikel germinal (GVBD) dan hanya 20% (4/20) dari oosit-oosit tersebut yang mencapai peringkat MII. Penemuan-penemuan ini menyokong data tentang perubahan sitoplasma bahawa COC, dan bukan CFO, yang dieramkan di antara 20 ke 30 jam adalah oosit yang mungkin akan menghasilkan kadar persenyawaan yang tinggi pada kaedah IVF kelak. Berdasarkan inilah hanya COC yang dieramkan selama 20 jam yang akan digunakan untuk kajian IVF berikutnya.

Kajian IVF ke atas COC yang diinseminasi dengan sperma sejuk-beku di dalam medium yang ditambahi dengan heparin dan diko-kultur dengan sel-sel epitelium oviduk menunjukkan bahawa 12.5% (7/56) oosit mencapai peringkat embrio 2-sel. Pengkulturan selanjutnya menghasilkan perkembangan seterusnya pada 5.3% (3/56) embrio-embrio ini kepada peringkat 4-sel. Oleh kerana tidak terdapat lagi perkembangan embrio preimplantasi selanjutnya yang melebihi peringkat 4-sel, nampaknya terdapat blok perkembangan di antara peringkat 4- dan 8-sel.

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ABBREVIATIONS AND SYMBOLS

The following abbreviations and symbols are used in the thesis:

ABEL	=	animal biotechnology-embryo laboratory
AI	=	artificial insemination
AV	=	artificial vagina
cAMP	=	cyclic adenosine 5-monophosphate
CG	=	cortical granules
cu	=	cumulus cells
co	=	corona cells
cy	=	cytoplasm
COC	=	cumulus-oocyte complex
CFO	=	cumulus-free oocyte
e.g.	=	exempli gratia (for example)
EM	=	electron microscope
ER	=	endoplasmic reticulum
ET	=	embryo transfer
FCS	=	foetal calf serum
FSH	=	follicle stimulating hormone
g	=	gram
GVBD	=	germinal vesicle breakdown
hr	=	hour
i.e.	=	id est (that is)
IVM	=	in vitro maturation
IVF	=	in vitro fertilization

IVC	=	in vitro culture
IVF-ET	=	in vitro fertilization and embryo transfer
IVMFC	=	in vitro maturation, fertilization and culture
J	=	junction
L	=	lipid bodies
LH	=	luteinizing hormone
LS	=	longitudinal section
M	=	Mole
MI	=	metaphase I
MII	=	metaphase II
mg	=	milligram
m	=	minutes
mi	=	mitochondria
ml	=	millilitre
mM	=	millimol
mm	=	millimetre
mv	=	microvilli
nm	=	nanometer
μm	=	micrometer
μl	=	microlitre
OL	=	oolemma
N	=	nucleus
Osm	=	osmol
P	=	cumulus cell processes
Pb1	=	polar body 1
Pb2	=	polar body 2

ps	=	perivitelline space
RNA	=	ribonucleic acid
TCM 199	=	tissue culture medium 199
TEM	=	transmission electron microscopy
TS	=	transverse section
V	=	vesicle
v/v	=	volume/volume
w/v	=	weight/volume
zp	=	zona pellucida
°C	=	degree celcius

LIST OF PRESENTATIONS AND PUBLICATIONS

Parts of the thesis have been presented and published or to be published as follows:

1. Yusoff, M., Rajikin, M.H., Ragunathan, T. and Abdullah, R.B. (1993). Fine structure of goat oocytes matured in vitro. *3rd. Sci. Conf. Electr. Micr. Soc.*, Malaysia, Penang, Nov. pp. **1-4.**

2. Rajikin, M.H., Yusoff, M. and Abdullah, R.B. (1994). The effects of freezing on the ultrastructure of goat sperm. *3rd. Symp. Appl. Biol.*, Melaka, pp. **19-21.**

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4. Rajikin, M.H., Embong, W.K.E. and Abdullah, R.B. (1995). Chromosomal studies of developing goat oocytes in vitro. *Anim. Reprod. Sci.* (submitted).

5. Rajikin, M.H. and Abdullah, R.B. (1995). In vitro studies of maturation and fertilization of oocytes and subsequent culture of embryos in vitro. *Theriogenology* (submitted).

(See Appendix 3)