EFFECT OF CUMULUS OOCYTE COMPLEX MORPHOLOGY ON *IN VITRO* MATURATION OF BOVINE OOCYTES*

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Abstract

An experimental design was framed to analyse the effect of cumulus oocyte complex (COC) morphology on in vitro maturation of bovine oocytes. Slaughter house derived bovine ovaries from South Indian breeds and crossbred cattle of Kerala were subjected to three retrieval methods to yield different quality grades of oocytes. Based on the number of layers of cumulus cells and ooplasm character, the oocytes were graded into four classes from A to D. Each grade of oocytes obtained through different retrieval methods were incubated at 38.5°C with 5% carbondioxide tension and maximum humidity for 24 h in TCM-199 medium supplemented with LH, FSH, estradiol, pyruvate and foetal calf serum. Maturation changes were assessed by cumulus expansion, formation of M II plates and polar body extrusion. Oocytes with more than three layers of cumulus cells exhibited maximum maturation rate irrespective of their retrieval method. The experiments revealed that COC morphology has a very significant role in maturation of bovine oocytes.

Key words: *Bovine oocyte, in vitro maturation, cumulus oocyte complex.*

In vitro maturation (IVM) is the starting point of a whole lot of biotechnological applications in animals like *in vitro* fertilisation (IVF), cloning, transgenic animal production and stem cell research. Another application of these techniques is for conservation of breeds and species that are on the verge of extinction. *In vitro* maturation and *in vitro* fertilisation techniques combined with marker assisted selection at embryonic stage will hasten the improvement of the production potential of cattle. Since cattle slaughter is banned in India except in Kerala and West Bengal, much work has not been carried out in Indian cattle in the field of IVM and IVF using oocytes derived from slaughter house ovaries. This experiment was designed to study the effect of cumulus oocyte complex morphology on *in vitro* maturation potential of bovine oocytes under different retrieval methods.

Materials and Methods

Slaughter house derived ovaries of South Indian breeds like Kangayam, Khillari, Hallikar and crossbred cattle of Kerala were used for the present IVM experiment. Ovaries were dissected out from animals within 30 to 60 min of slaughter, and transported to the laboratory within 2 to 4 h in freshly prepared normal saline solution fortified with 100 IU/ml Benzyl penicillin (Alembic Ltd. Vadodara, India) and 100 µg/ml Streptomycin sulphate (Alembic Ltd. Vadodara, India) maintained at 36 to 38°C. Oocytes were retrieved from ovaries by retrieval methods namely aspiration, slicing and puncture. The retrieval process was carried out in hydroxy ethyl piperazine ethane sulfonic acid (HEPES) buffered Tyrode's lactate medium enriched with bovine serum albumin (BSA) @ 0.6% and maintained at 37°C. Heparin was supplemented to this medium @ 0.1 mg/ml.

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Classification of Cumulus - Oocyte Complexes. (200X)

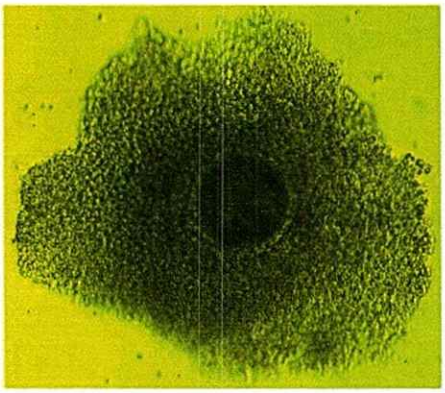


Fig. 1. Class A oocyte

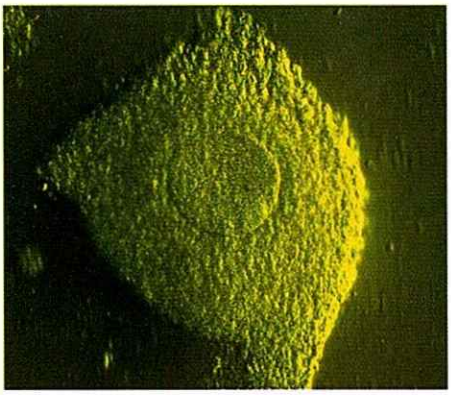


Fig. 2. Class A oocyte

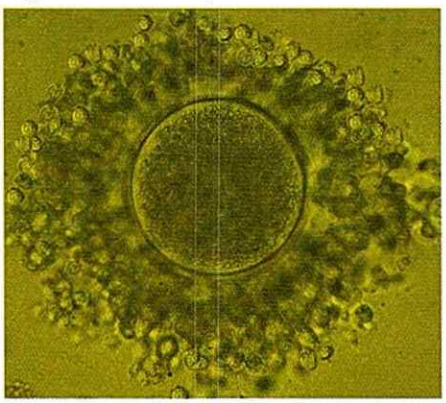


Fig. 3. Class B oocyte

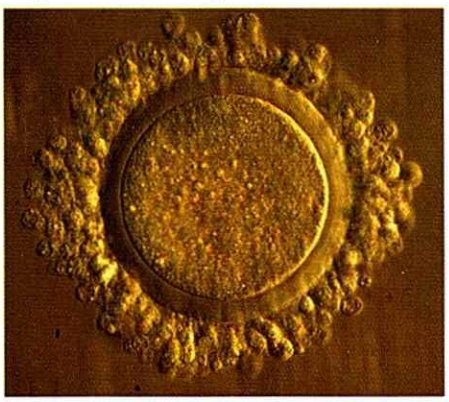


Fig. 4. Class C oocyte

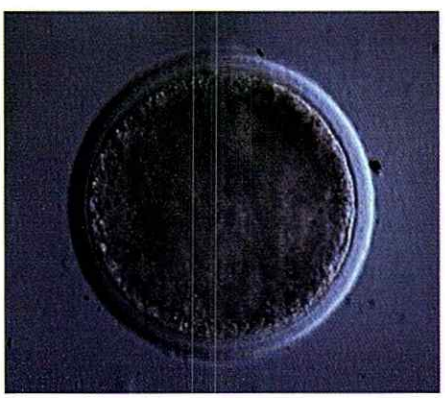


Fig. 5. Class D oocyte

Fig. 6. Class D oocyte

Based on the number of layers of cumulus cells and ooplasm character, the oocytes were graded into four classes namely A,B,C and D. Class A oocytes were characterised with more than five complete layers of cumulus cells and uniform granulation of ooplasm (Figs. 1 & 2), Class B with 3 to 5 complete layers of cumulus cells and uniform granulation of ooplasm (Fig. 3), Class C with 1 to 2 complete layers of cumulus cells and uniform granulation of ooplasm (Fig. 4), and Class D as denuded oocytes with uniform granulation of ooplasm (Figs. 5 & 6).

Medium used for maturation of oocyte was freshly prepared TCM-199 enriched with follicle stimulating hormone (FSH)@ 0.5 µg/ml (Folltropin – V, Vetrepharm Canada Inc), luteinising hormone (LH) @ 5 µg/ml (Lutropin % V, Vetrepharm Canada Inc), estradiol @ 1 µg/ml, sodium pyruvate @ 0.2 mM and foetal calf serum (FCS) 10%. Culture condition set for this study was 38.5°C , 5%

Matured Bovine Oocytes – Extruding First Polar body (400X)

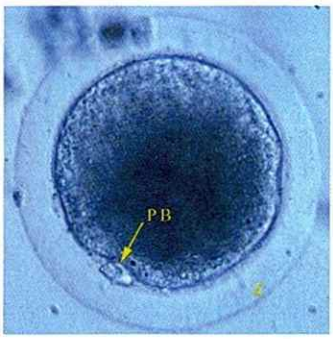


Fig. 7. & Fig. 8. – Matured oocytes with extruded polar body
PB – Polar body, Z - Zona Pellucida, PS – Perivitelline space

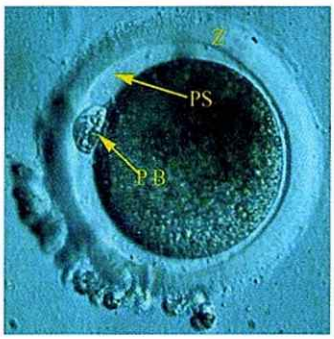


Fig.9. – Mature oocytes with perivitelline space

carbondioxide tension and maximum humidity. Standard water-jacketed type CO_2 incubator (Lab line instruments Inc, USA) was used to achieve this culture environment. After 24 h of culture, all oocytes in the culture drops were examined under zoom stereo microscope (Leica MZ-6, Leica micro systems, Germany) for maturation associated changes such as expansion and mucification of cumulus cells. The oocytes were denuded by vortexing and examined for extruded polar bodies (Figs. 7, 8 & 9). All the denuded oocytes were stained with 1% aceto-orcein (with 1% orcein in 45% acetic acid), and then examined for nuclear changes associated with maturation. Nuclear stages were identified as germinal vesicle stage (GV), germinal vesicle breakdown stage (GVBD), metaphase I stage (M I) and metaphase II stage (M II) with extruded polar body (Figs. 10 to 15). Oocytes with extruded polar body and M II plates were counted as mature. Data on cumulus

Nuclear maturation – Sequential changes (400X)

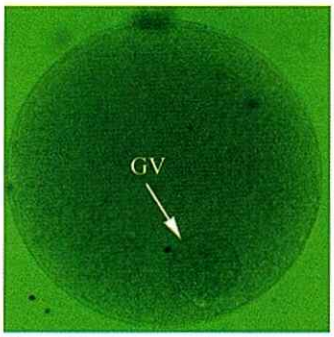


Fig. 10. Oocyte showing germinal vesicle (GV) stage. Nuclear membrane is visible

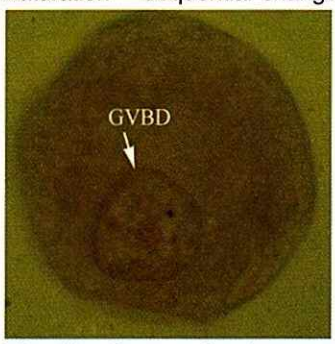


Fig. 11. Ooctye showing germinal vesicle break down stage (GVBD)

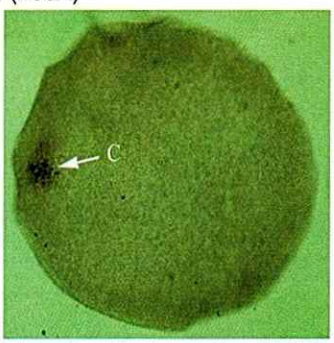


Fig. 12. Ooctye after GVBD, with condensed chromatin mass (C).

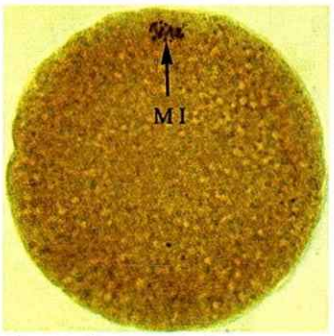
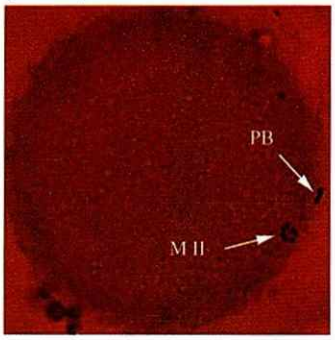


Fig. 13. Ooctye with chromosomes arranged in equatorial plane at metaphase I (M I Stage)



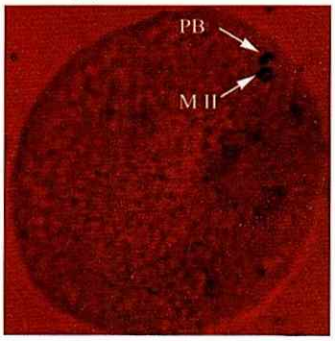


Fig. 14. & 15. Ooctyes showing metaphase II (M II stage) and extruded polar body (PB) under aceto-orcein staining (400X).

expansion and nuclear maturation of different grades of oocytes were analysed using *Chi*-square analysis and those data showing significant difference ($P \leq 0.05$) were subjected to pair wise data analysis using *Chi*-square test.

All chemicals and Media used in this study were from Sigma Chemicals St. Louis, USA, unless mentioned otherwise.

Results and Discussion

Analysis of data revealed that class A oocyte did not show any significant difference in maturation rate compared to class B oocytes in all the methods except slicing. But significantly lower maturation rate was observed for class C oocytes in comparison to classes A and B in all methods (P d" 0.05). Class D oocytes failed to mature in all methods of retrieval. Results are expressed in detail in tables 1 and 2.

Maturation rate for class A oocytes obtained by aspiration method in this study was slightly lower than the results of Tatemoto and Terada (1995) and Konishi *et al.* (1996). Leibfried and First (1979) when employed the same method and same class of oocytes for maturation studies, a lower maturation rate was obtained in bovines. Maturation rate reported by Choi *et al.* (1998) and Rodriguez and Farin (2004) were much higher than in the present study, when bovine oocytes were used for the experiments.

Class A and B oocytes did not differ significantly in polar body extrusion rate. But polar body extrusion rate of class C oocytes was significantly lower than class A and B oocytes. Out of nine class D oocytes examined none exhibited nuclear maturation. The result obtained by Carolan *et al.* (1992) with regard to slicing was slightly higher than the maturation rate of class A oocytes in the present study. Much lower maturation rate was obtained by Arlotto *et al.* (1996) for class A oocytes retrieved by slicing.

Maturation rate of class A oocytes in puncture method was comparable to the results of Schellander *et al.* (1990) in cattle. Statistical analysis revealed that highest maturation rate was observed in class A oocytes, followed by class B. Classes A and B oocytes exhibited significantly higher polar body extrusion rate than class C. Classes A and B did not differ significantly in polar body extrusion rate.

Results of the study clearly indicates that the morphology of COC have significant effect on maturation of bovine oocytes. Greater the number of cumulus layers greater was the maturation rate. Oocytes with more than three layers of cumulus cells exhibited significantly higher maturation rate than the denuded oocytes and oocytes with partial cumulus layers. These results agreed with the results of Leibfried and First (1979) and Konishi *et al.* (1996). Raghu *et al.* (2002) opined that removal of cumulus cells

Table 1. Effect of quality of cumulus oocyte complexs(COCs) on cumulus expansion rate

Sl. No.	Retrieval technique	Grade of oocyte	Oocyte kept for maturation	Oocyte showing cumulus expansion	Expanded COCs (%)
1	Aspiration	A	130	108	83.08[a]
		B	82	56	68.29[a]
		C	76	34	44.74[b]
		Overall	288	198	68.75
2	Slicing	A	198	138	69.70[c]
		B	158	84	53.16[d]
		C	102	36	35.29[e]
		Overall	458	258	56.33
3	Puncture	A	162	114	70.37[f]
		B	116	72	62.07[f]
		C	78	30	38.46[h]
		Overall	356	216	60.67

Percentage values bearing different superscripts in same column differs significantly within retrieval methods (P≤ 0.05).

Table 2. Effect of quality of oocytes on nuclear maturation rate

Sl. No.	Retrieval technique	Grade of oocyte	Number of oocytes examined	Oocytes showing Metaphase II plates	Oocytes showing polar body extrusion	Percentage of nuclear maturation	Percentage of polar body extrusion
1.	Aspiration	A	44	36	20	81.8[a]	45.5[a]
		B	28	16	8	57.1[a]	28.6[a]
		C	26	10	4	38.5[b]	15.4[b]
2.	Slicing	A	38	30	16	78.9[c]	42.1[c]
		B	24	10	6	41.7[d]	25.0[d]
		C	14	4	2	28.6[d]	14.3[d]
3.	Puncture	A	50	40	22	80.0[e]	44.0[e]
		B	38	20	12	52.6[e]	31.6[e]
		C	26	8	4	30.8[f]	15.4[f]

Percentage values bearing different superscripts in same column differs significantly within retrieval methods (P≤ 0.05)

perturbed the cytoplasmic maturation and hence developmental competence was reduced.

Cumulus cells communicate to the oocyte across zona pellucida through corona radiata cells, which penetrate the zona pellucida and form gap junctions with oolemma. These intercellular communications allow metabolic transfer as molecules of small molecular weight help in nutrition of oocytes, which ultimately plays a vital role in oocyte growth and maturation (Buccione *et al.*, 1990; Armstrong *et al.*, 1996). Staigmiller and Moor (1984) opined that granulosa cells provide energy substrate, some amino acids, nucleotides and phospholipid precursors to the oocyte, that generate some interactional signals which influence the nucleus and direct the synthesis of certain structural proteins and maturation specific proteins.

Lorenzo *et al.* (1994) observed both epidermal growth factor (EGF) and insulin like growth factor (IGF-I) alone or together stimulated nuclear maturation in immature bovine oocytes and opined that their beneficial effect was mediated through cumulus cells. Receptors for EGF have been demonstrated in bovine cumulus and small antral granulosa cells and the number of EGF binding site has been reported to be influenced by gonadotrophins. Epidermal growth factor exerts its stimulatory effect by binding to granulosa cells since this phenomenon was seen only in cumulus enclosed oocyte, and not in denuded oocyte (Chauhan *et al.*, 1999). Insulin like growth factor II with FSH synergistically enhanced DNA synthesis, protein synthesis and steroidogenesis in the presence of granulosa cells. The synergistic effect was mainly caused by increase of IGF-II receptors in granulosa cells by FSH (Pawshe *et al.*, 1998).

As per Guoliang *et al.* (1994) oocyte cumulus connections were crucial as far as initiating production of a meiosis inducing substance was concerned. Glutathione is another important substance needed for maturation and transported to oocyte through cumulus cells. Byskov *et al.* (2002) reported that cumulus cells were needed for production of maturation promoting factor (MPF). van den Hurk and Zhao, (2005) also reported the beneficial effect of MPF on the onset of oocyte maturation.

So the result of this experiment is in confirmation with the view that the cumulus oocyte complex morphology has a definite role in the *in vitro* maturation of bovine oocytes. Oocytes with multiple layers of cumulus cells (three or more layers) matured better than the denuded oocytes or oocytes with lesser number of cumulus cells (less than three layers). Oocytes with more than three complete layers of cumulus cells (classes A and B) are best suited for *in vitro* maturation in cattle.

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