



Original Research

In vitro Development Competence of Buffalo Oocytes: Effect of Oocytes Quality on Maturation, Embryonic Developments and Apoptosis

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Abstract

The present study undertaken to assess the effect of different grades of cumulus-oocyte complexes (COC's) on in vitro maturation and embryonic development along with apoptosis changes by confocal microscope. The A and B grade of COC's were recovered from slaughterhouse derived ovaries on the basis of cumulus mass. A total 1229 COC's were recovered from 428 ovaries, out of which 25.14% were of grade A and 44.18% of grade B. In vitro fertilization of grade A COC's produced significantly higher rate of cleavage and blastocyst ($89.99 \pm 1.49\%$ and $41.99 \pm 1.82\%$) as compared to grade B ($81.33 \pm 0.81\%$ and $30.66 \pm 1.63\%$). The low rate of embryonic development in grade B COC's might be due to higher incidence of apoptosis (36.66 ± 4.31 vs 26.66 ± 2.63) as compared to grade A as observed by Annexin-V/Propidium iodide staining under confocal microscope. Thus, our study concluded that the cumulus mass had significant impact on different developmental processes of oocytes, fertilization and embryonic developments.

Key words: Apoptosis, Buffalo, Cumulus-Oocyte Complex's (COC's), In vitro Fertilization

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Introduction

In vitro maturation (IVM) and in vitro fertilization (IVF) of oocytes obtained from slaughterhouse derived ovaries have recently provided a low cost technology for producing large number of embryos for animal research. The outcome of in vitro maturation of oocytes recovered from abattoir derived ovaries is highly variable (Galli *et al.*, 2003; Hammam *et al.*, 2010) depending on availability of quality follicular oocytes, which has a significant impact on fertilization and embryo development. Cumulus cells of cumulus-oocyte complexes (COC's) play important role during in vitro maturation and oocyte competence for embryonic development (Mori *et al.*, 2000), as they are responsible for nurturing oocyte growth, development and





gradual acquisition of oocyte developmental competence (Downs, 2001; Xia *et al.*, 2000). Thus, oocyte quality is considered as key factor that determine the embryo growth and apoptosis during embryonic development. Apoptosis is defined as self-destruction of cells under physiological control (Ameisen, 2002). Typical features of apoptotic cells include cell shrinkage, translocation of phosphatidylserine (PS) to the outer cytoplasmic membrane, DNA fragmentation, and segmentation of the cells into apoptotic bodies (Yang and Rajamahendran, 2002). PI does not stain live or early apoptotic cells due to the presence of an intact plasma membrane (Darzynkiewicz *et al.*, 1992). In late apoptotic and necrotic cells, the integrity of the plasma and nuclear membranes decreases (Kroemer *et al.*, 1998; Denecker *et al.*, 2001), allowing PI to pass through the membranes, intercalate into nucleic acids, and display red fluorescence. Further, the apoptotic changes in early fertilized embryos were analyzed after staining with the Annexin V/Propidium iodide (Annx-PI) and observed under confocal microscope. Hence, the objective of the present study was to investigate the developmental competence of slaughterhouse derived buffalo oocytes of different quality in terms of *In vitro* maturation, fertilization, embryonic development and apoptosis.

Materials and Method

All the chemicals and media were purchased from Sigma Chemicals Co. (St Louis, MO) and disposable plastic wares were from Nunc (Roskilde, Denmark), unless mentioned specifically.

Oocyte Recovery and Grading

Buffalo ovaries were collected from large animal slaughterhouse in pre equilibrated saline solution (supplemented with gentamicin 50µg/ml) aseptically. The ovaries were trimmed and washed 4-5 times with Dulbecco's phosphate buffer saline (DPBS). Aspiration of COC's from 3-8mm sized ovarian follicle by sterile 18 gauge syringe having 2-3ml of DPBS solution under aseptic conditions. Grading of oocytes were carried out under stereo zoom microscope (Olympus, Japan) in a 90mm petri disc. Recovered oocytes were graded from A and B (Gordon, 1995) as per the following criteria: (i) Grade A- Oocytes with more than three layers of compact cumulus cells layers and evenly granular homogenous ooplasm. (ii) Grade B: Oocytes with two or three layers of compact cumulus cells layers and evenly granular homogenous cytoplasm (Fig. 1A and B).

In vitro Maturation (IVM)

In vitro maturation was done in maturation media consisting of TCM-199 fortified with 10µg/ml follicle stimulating hormone (FSH), leutinizing hormone (LH), 1µg/ml estradiol, 7.5% (v/v) fetal bovine serum (FBS), 50µg/ml gentamicin and 0.8mM/ml sodium pyruvate. The maturation of oocytes was carried out in groups of 15-20 oocytes per droplet of 50µl. The droplets were overlaid with sterile pre-equilibrated mineral oil (COOK®, Australia) and kept under humidified (99%) atmosphere for 24 hours in CO₂ (5%) incubator

at 37°C. The evaluation of matured oocytes was done on the basis of cumulus expansion and extrusion of primary polar body (Fig. 1C).

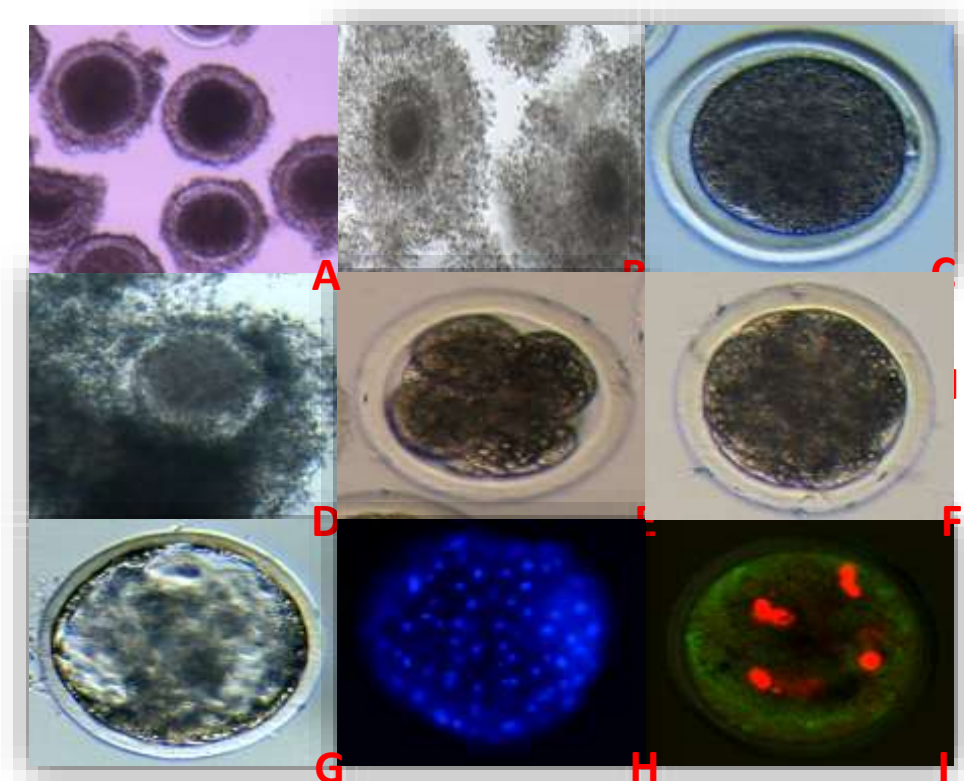


Fig. 1: Figure showing *in vitro* maturation, fertilization, embryonic development and apoptosis: (A). Different grades of Immature COCs (100X) (B). Matured COCs (200X) (C). Oocyte showing primary polar body (200X), (D). Co-incubation of oocyte with capacitation sperm (200X) (E). 8 cell stage (200X), (F). Compact morula (200X), (G). Blastocyst (100X), (H). Hoechst stained embryo showing nucleus (100X) (I). Stained embryo showing annexin V (green) and PI (red) under confocal microscope.

Sperm Capacitation and *In vitro* Fertilization

The sperms for *in vitro* maturation were collected from slaughterhouse derived epididymis and processed under aseptic conditions in laboratory as described earlier for ovaries. The, sterile epididymus was incised with sterile blade and exudated sperms were collected in 10ml Bracket and oliphant (BO) media supplemented with caffeine sodium benzoate (1mM). Initially, the concentrations, morphology, initial and gross motility of sperm were examined under inverted microscope. The sperm suspension (in BO media) was centrifuged twice at 1000rpm for 8min. The sperm pellet was resuspended in 5ml BO fertilization media (supplemented with 1% BSA+ heparin, 50µg/ml) and centrifuged at 1000rpm for 5min. Finally the pellet was suspended in 1ml of BO fertilization media and centrifuged at 1000rpm for 1min. The pellet was loosened with a fine bore Pasteur pipette to allow the sperm movement upward for swim up and the tube was kept at inclined position inside the CO₂ incubator for 30min. During this period, matured oocytes of

both grades (A and B) were transferred into the 50µl droplets of BO fertilization media at the rate of 15-20 oocytes per droplets. After incubation of 30min, the sperms were taken from the top layer and added into the fertilization droplets to achieve a final concentration of 10^6 sperm/ml. The sperm and oocytes were co-incubated at 38.5°C in CO₂ (5%) under humidified atmosphere for 18h. After co-incubation, the oocytes were denuded using 0.1% hyaluronidase and cultured Research vitro cleavage media (RVCL, COOK®, Australia) upto blastocyst formation and embryos of each grade were assessed for developments into different embryonic stages and degree of apoptosis was examined under confocal microscope (Fig. 1D, E, F and G).

Blastomere Count

The blastocysts of both groups were stained with Hoechst 33342 as described by Kumar *et al.* (2014). In brief, the blastocysts were washed 3-4 times in PBS supplemented with 1mg/ml polyvinylpyrrolidone (PVP) and subsequently transferred to a 50µl drop of Hoechst 33342 stain having 10µg/ml in PBS for 10-15 min in a dark chamber. Finally, embryos were kept on a sterile glass slide and covered with cover slip for blastomere count under fluorescence microscope (Fig. 1H).

Assessment of Apoptosis in Early Embryos by Annexin V- Propidium Iodide (Annx-PI) Staining

After 72h of fertilization, embryos were stained by using annexin V FITC apoptosis kit, as per manufacturer's instructions (ApoDETECT™ ANNEXINV-FITC Kit, Invitrogen™, Cat-33-1200). Briefly, embryos were washed twice in PBS (pH-7.4) and subsequently suspended in 500µl of 1X binding buffer kept in a well of 4 well plate. Embryos were then transferred into a second well containing 10µl of Annexin V-FITC +190µl 1x binding buffer and incubated for 10min at room temperature. These embryos were washed with 1X binding buffer and finally resuspended in 10µl propidium iodide (20µg/ml) + 190µl of binding buffer. The samples were kept on micro confocal dish (Ibidi, GmbH, Germany) and apoptosis were evaluated by Annexin V (FITC, 495-519nm) and propidium iodide (PI, 537-619nm) under confocal microscope (FV10i, Olympus, Japan). The green signal emitted by annexin V was an indicator of early apoptosis while red fluorescent light emitted by propidium iodide is indicative of late apoptosis or necrosis (Fig.1I).

Statistical Analysis

Data generated from the study was analyzed by using student's t test (Snedecor and Cochran, 1994).

Results and Discussion

In vitro Maturation (IVM) of COC's from Slaughterhouse Derived Buffalo Ovaries

A total 1229 COC's were aspirated from 428 ovaries under 12 experimental trials. For *in vitro* maturation of oocytes a total of 309 (25.14%) grade A and 543 (44.18%) grade B COC's were taken under all the

experimental trials. The result as shown in Table 1 revealed that the grade A oocytes had significantly higher ($P \leq 0.05$) maturation rate ($93.33 \pm 0.896\%$) as evidenced by primary polar body extrusion as compared to grade B oocytes ($85.87 \pm 0.816\%$).

Table 1: *In vitro* maturation of immature COC's from slaughterhouse derived buffalo ovaries

S. No.	No. of Ovaries	Aspirated Oocytes	Grade- A	Grade-B
1	428	1229 (2.87/ovary)	309 (25.14%)	543 (44.18%)
			Oocytes Showing Polar Body	
			288 (93.33 ± 0.896^a)	466 (85.87 ± 0.816^b)

Values within parentheses indicate mean $\pm$ SE%; Values with different superscripts in column differed significantly at $P \leq 0.05$.

Embryonic Development of *In vitro* Fertilized Grade A and Grade B Oocyte Groups

Out of total 10 experimental trials for *in vitro* fertilization, 5 trials were conducted each for grade A and B consisting of total 150 oocytes. Our result showed that the developmental rate of embryos under group A was significantly higher ($P \leq 0.05$) in terms of 2-4 cells (89.99 ± 1.49 vs $81.33 \pm 0.81\%$), 8-16 cells (77.33 ± 2.79 vs $67.33 \pm 1.24\%$), morula (59.99 ± 1.05 vs $51.33 \pm 1.68\%$) and blastocysts (41.99 ± 1.82 vs $30.66 \pm 1.63\%$) as compared to embryos developed from grade B oocytes, however the mean blastocyst count in blastomere were non-significant ($P \leq 0.05$) in both groups (160.48 ± 0.94 and 158.88 ± 1.16) (Table 2).

Table 2: Embryonic developmental competence of different grades of oocytes after *In vitro* fertilization of buffalo oocytes

		Embryonic development stages (Mean $\pm$ SE%)				Average no. of blastomere in blastocysts (Mean $\pm$ SE)
No. of oocytes		2-4 cells	8-16 cells	Morula	Blastocyst	
Grade A		135	116	90	63	160.48 ± 1.24^a
	150	(89.99 ± 1.49^a)	(77.33 ± 2.79^a)	(59.99 ± 1.05^a)	(41.99 ± 1.82^a)	
Grade B		112	101	77	46	158.88 ± 1.16^a
	150	(81.33 ± 0.81^b)	(67.33 ± 1.24^b)	(51.33 ± 1.68^b)	(30.66 ± 1.63^b)	

Values with different superscripts within column differed significantly at $P \leq 0.05$.

Apoptotic Evaluation of *In vitro* Produced Buffalo Embryos

Out of total 10 experimental trials, 5 trials were conducted each for grade A and B consisting of 30 oocytes each. As shown in Table 3 the apoptotic rate in grade B embryos were significantly higher ($P \leq 0.05$) as compared to grade A embryo (36.66 ± 4.31 vs $26.66 \pm 2.63\%$).

Table 3: The percentage of embryos showing apoptotic morphology stained with Annex-PI

Grade of oocytes	Total no. of embryos stained	Apoptosis rate in early embryos (%)
Grade A	30	8 (26.66 ± 2.63^a)
Grade B	30	11 (36.66 ± 4.31^b)

Values within parentheses indicate mean $\pm$ SE%; Values with different superscripts in column differed significantly at $P \leq 0.05$.



The impact of high quality of oocyte on embryonic development competence has been emphasized by many scientists (Karami-Shabankareh, 2012; Galli *et al.*, 2003 and Hammam *et al.*, 2010). Morphologically, the quality and developmental potentials of the oocytes primarily depend on layers of cumulus mass associated to the oocytes (Ebner *et al.*, 2003; Boni, 2012). However, the correlation between cumulus cells and oocyte maturation is not yet fully established. Our study revealed that the oocytes with more layers of cumulus mass (grade A) showed significantly higher developmental competence in all developmental parameters as compared to the oocytes with less cumulus mass (grade B). Our findings are in line with Eppig (1989) who reported that the good cumulus mass had beneficial impact on oocyte maturation and embryonic development. As per De Souza *et al.* (2013) the cumulus cells secreted factors or proteins into the media to maintain the gap junction communication between cumulus cells and oocytes, thus enriched the environment for optimal fertilization. Cumulus cells have a critical role to play in mammalian follicular control and the regulation of oogenesis, ovulation rate and fecundity (McNatty *et al.*, 2004; Gilchrist and Thompson, 2007). The cumulus cells maintain a high concentration of reduced glutathione in oocyte which facilitate the processing of sperm chromatin configuration changes after fertilization (De Matos *et al.*, 2002). There are evidences that the cumulus cells protect the oocytes against oxidative stresses during oocyte maturation (Tatemoto *et al.*, 2000). The antioxidant actions of cumulus cells might contribute in embryotrophic properties toward fertilization and embryonic development.

Fragmentation is one of the hallmarks of apoptosis (Hardy, 1999), it could also be used as a non-invasive marker of embryonic apoptosis. Nuclear fragmentation and condensation are key morphological elements of apoptosis and are necessary to confirm biochemical assessments of apoptosis (Hardy, 1999; Gjørret *et al.*, 2003). Studies using arrested fragmented early cleavage embryos, morphological and biochemical characteristics of apoptosis were detected by using TUNEL and annexin V staining (Jurisicova *et al.*, 1996; Levy *et al.*, 1998). Most of the previous studies had used TUNEL assay which stained only fragmented DNA, which detect the last stage of the apoptosis process. The use of Annx-PI in our study was meant to detect phosphatidylserine on the outer leaflet of the bilayer plasma membrane as it is the first event of the apoptosis process. In the present study, data revealed that grade B embryos have significantly higher apoptotic rate as compared to grade A embryo, which is in accordance to embryonic developmental rate of grade A and grade B oocytes. The relevant literature for comparison of our findings could not be traced in buffaloes however, Matwee *et al.* (2000) reported that DNA fragmentation was not observed in early stage of bovine embryos (less than 8-cell stage), it was observed in the oocyte, morula and blastocyst.

Conclusion

It can be concluded from our study that the morphologically good quality of COC's (grade A) had a higher developmental competence for maturation, fertilization and embryonic developments as compared to



oocytes having less number of cumulus rings. Further, the apoptotic events in the early embryos developed by grade A oocytes was significantly lower in comparison to the embryos produced by grade B oocytes. These differences in developmental potential and embryo viability between different oocytes groups might be due to the embryotrophic effects of cumulus cells in the oocytes.

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