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Short communication

Effect of cattle oocyte quality on pronuclear formation and subsequent embryo development

(Kesan kualiti oosit lembu terhadap pembentukan pronukleus dan perkembangan embrio seterusnya)

B. Habsah*, K. Musaddin* and I. Mohd Rafi**

Keywords: pronuclear formation, blastocyst, cumulus-oocyte complex, in vitro fertilization, cleavage, cattle embryo

Abstract

The effect of oocyte quality on the pronuclear (PN) formation and subsequent development of embryos in local cattle was studied. Cattle oocytes matured and fertilized in vitro were unable to acquire developmental competence unless matured with intact cumulus cells. The presence of cumulus cells promoted normal fertilization with proper pronuclear (2PN) formation. In the present study, the characteristics and quality of cumulus-oocyte-complexes influenced the pronuclear formation but not subsequent cleavage and blastocyst formation. The oocytes from Grade A, Grade B and Grade B' were capable of fertilization and development under in vitro conditions. Fertilization rates were significantly different (p <0.05) among the three grades of oocytes. Grade A had the highest fertilization rates (55.0%) followed by Grade B (44.0%) and Grade B' (30.2%). Incidence of polyspermy (>2PN) was 18.8% in Grade B' oocytes but none in Grades A and B oocytes. The mean percentages of cleavage and blastocyst rates were not significant (p >0.05) in Grades A, B and B' (71.6, 74.9 and 73.6%, respectively, and 10.5, 10.4 and 7.4%, respectively). In conclusion, this study indicated that the compactness of cumulus cells surrounding the oocytes influenced the pronuclear formation of cattle oocytes but not cleavage and blastocyst rates.

Introduction

The importance of intact cumulus cells for developmental competence of immature oocytes has been well established (Shioya et al. 1988; Fukuda et al. 1990). Oocytes matured without cumulus cells frequently showed deficiencies in pronuclear formation that led to low frequency of cleavage (Vanderhyden and Amstrong 1989). Similar deficiencies have been reported in rabbits (Thibault 1977), hamsters (Leibfried and Bavister 1983), mice (Schroeder and Eppig 1984), pigs (Mochizuki et al. 1991) and cattle (Leibfried-Rutledge et al. 1987).

These authors suggested that the developmental problem of oocytes is due to deficient in cytoplasmic maturation. Although serum is able to enhance the

*Strategic Livestock Research Centre, MARDI Headquarters, Serdang, P.O. Box 12301, 50774, Kuala Lumpur, Malaysia

**Faculty of Educational Studies, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia

Authors' full names: Habsah Bidin, Musaddin Kamaruddin and Mohd Rafi Ishak

E-mail: habsahb@mardi.gov.my

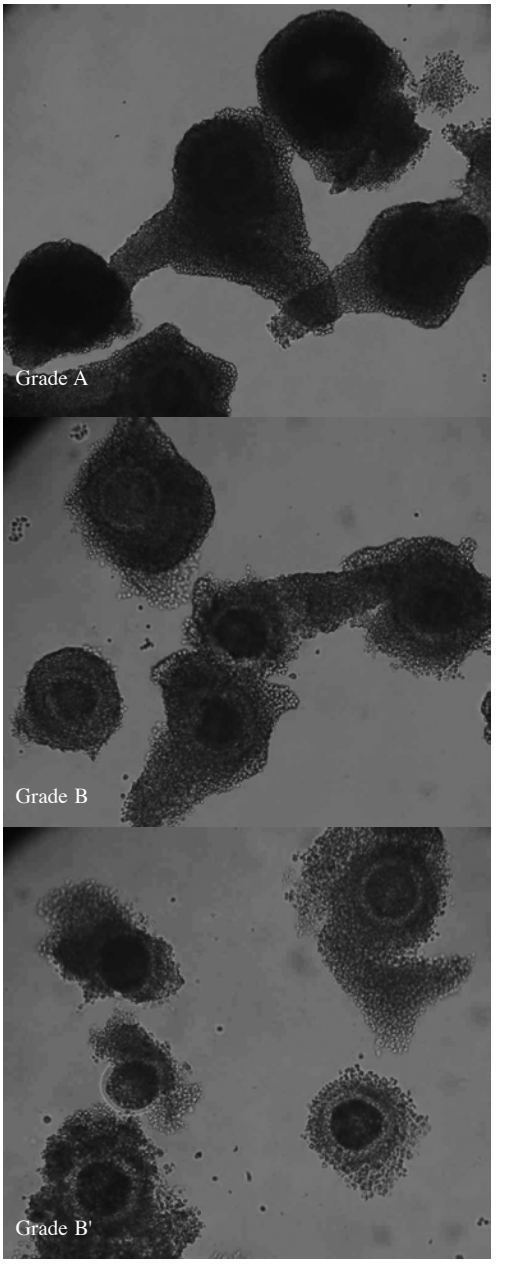
penetrability of cumulus-free oocytes, the presence of cumulus cells even in the absence of serum promotes normal fertilization with proper pronuclear formation. Besides controlling the rate of nuclear maturation and helping maintain penetrability of oocytes, the presence of cumulus cells seems necessary for promoting normal cytoplasmic maturation. Therefore, the present study was conducted to assess the pronuclear formation and subsequent development of three different quality grades of oocytes matured, fertilized and cultured under in vitro conditions.

Materials and methods

Slaughterhouse ovaries were transported to the laboratory in Dulbecco's Phosphate Buffer Saline (D-PBS) at 30–37 ºC within 2 h of slaughter. The cumulus-oocyte-complexes (COCs) were harvested from follicles by slicing the ovaries and were morphologically classified according to the quality or compactness of the cumulus cells: Grade A (compact and dense cumulus cell layers), Grade B (compact but not dense cumulus cell layers) and Grade B' (partially naked with thin cumulus cell layers or small remnants of cumulus cells) categories (*Plate 1*).

The COCs were washed twice in collection medium (PBS + 10% steer serum) and once in maturation medium consisting of Tissue Culture Medium 199 (TCM-199), 1.25 μl/ml gentamycin solution, 20 mM sodium pyruvate, 100 mM L-glutamine, 1 μg/ml oestradiol-17β and 25% steer serum (Habsah et al. 1999). The COCs were matured in a maturation medium for 20–24 h in a humidified atmosphere at 38.5 °C and 5% carbon dioxide (CO_2).

Frozen thawed Mafriwal semen was used to fertilize the oocytes. Spermatozoa were washed twice in Brackett-Oliphant (BO) medium, which was supplemented with 20 mg/ml heparin. The concentration of spermatozoa was adjusted and diluted in half with a mixture of BO and 20 mg/ml Bovine Serum Albumin (BSA) before



Grade A: oocytes with compact and dense cumulus cell layers
Grade B: oocytes with compact but not dense cumulus cell layers
Grade B': oocytes with thin or with small remnants of cumulus cell layers or partially naked oocytes

Plate 1. Categories of oocytes used for IVMFC (100x magnification)

5 µl/ml calcium-ionophore A23187 was added. Matured COCs were fertilized with 1 x 10^6 sperm per ml in BO medium droplets under mineral oil for 18–20 h in a humidified atmosphere at 38.5 °C in a 5% CO_2 incubator.

In Experiment 1 (n = 222), following the COCs-spermatozoa co-incubation, presumptive zygotes were vortexed in 3% trisodium citrate (BDH Limited, Poole, England) for 4 min to remove all cumulus cells before fixing in 3:1 methanol:acetic acid at 4 °C for 24 h. The fixed zygotes were stained with 1% aceto-orcein for the determination of pronuclear formation under a phase contrast Zeiss microscope at 400x magnification. Oocytes that were arrested at metaphase II (MII) or with one pronucleus (1PN) were considered unfertilized and those with two pronuclei (2PN) or three pronuclei (3PN) were considered fertilized (Musaddin 1998).

In Experiment 2 (n = 1,818), 18–20 h after insemination, the oocytes were washed twice in BO medium supplemented with 10 mg/ml BSA and twice in Charles Rosenkranz 1 + amino acid (CR1aa) medium prior to transfer to CR1aa microdroplets (Habsah et al. 1999). The embryos were examined under an inverted microscope for cleavage and subsequent development for 7 days. Analysis of Variance and Duncan Multiple Range Test were used to test the significance of the parameters measured.

Results

The photomicrographs of some of the stages during pronuclear development are shown in *Plate 2*. Significantly higher (p <0.001) number of Grade B' oocytes (40.00%) were arrested at MII compared with Grades B (24.31%) and A (21.25%). The mean percentage of oocytes with 1PN and with unidentified chromosomes in Grades B',

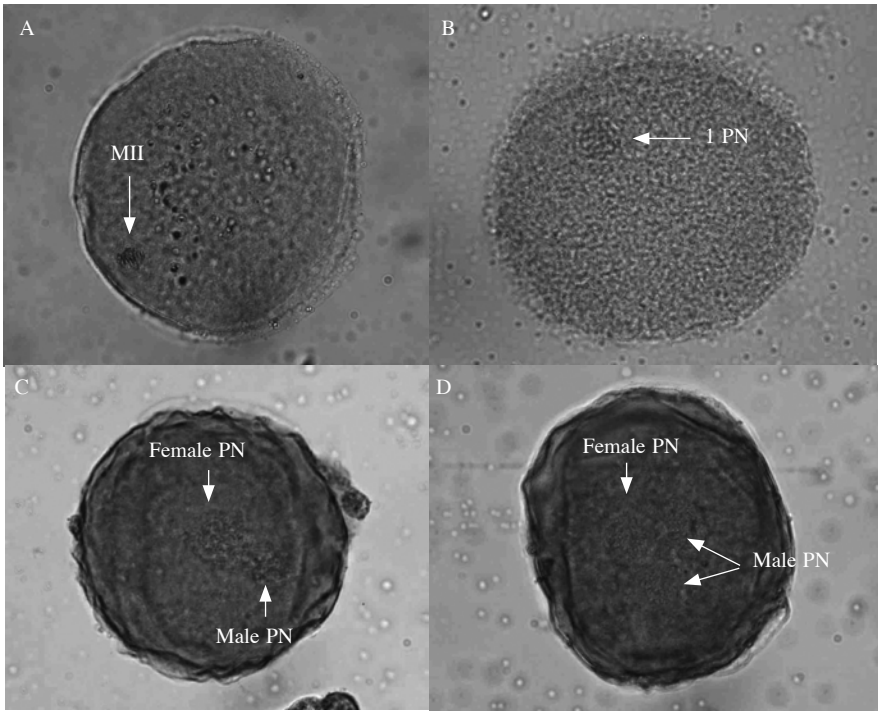


Plate 2. Photomicrographs of some of the stages during pronuclear development (A=MII, B=1PN, C=2PN, D=3PN) after 18 to 20 hours of insemination (400x magnification)

B and A were 25.0, 20.0 and 24.58%, respectively and 36.19, 29.17 and 27.78%, respectively. The differences between the mean percentages were not significant (*Figure 1*).

In terms of pronuclear development in Grades A and B oocytes, the fertilization rate based on oocytes with 2PN was significantly higher (p <0.05) compared with other pronuclear stages such as MII, 1PN and unidentified values. No incidence of polyspermy (3PN) was observed in both grades. However, in Grade B' oocytes, significant differences (p <0.05) were observed in MII and 1PN and in MII and 3PN. In Grade B' oocytes, higher MII value was observed followed by unidentified, 2PN, 1PN and 3PN.

The mean percentage of cleavage rate and blastocyst rate of the three grades of oocytes is shown in *Table 1*. No significant differences were observed in all grades of oocytes. The mean percentage of cleavage rates in Grades A, B and B' were 71.58, 74.94 and 73.57%, respectively, while the mean percentage of blastocyst rates were 10.47, 10.43 and 7.43%, respectively. The mean percentage of developmental stages of all grades of oocytes were significantly different (p <0.05), except at morula and compact morula stages in Grade A oocytes (28.87% vs. 21.65% for morula and compact morula, respectively). Among all grades of

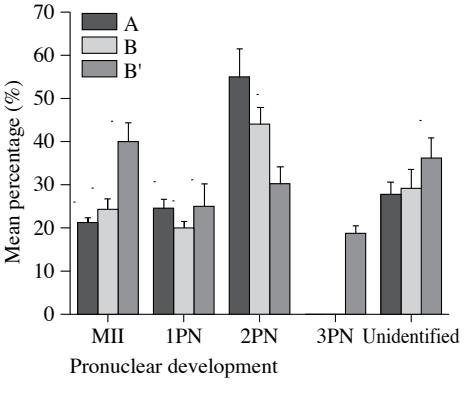


Figure 1. Mean percentage and standard error of pronuclear development of three grades of oocytes (Grades A, B and B')

Table 1. Mean percentage and standard error of different developmental stages in the three grades of oocytes

Classification of oocytes	Number of oocytes	Developmental stage of oocytes (mean ± S.E.)						
		2-cell	4-cell	8-cell	16-cell	Morula	Compact morula	Blastocyst
A	506	71.58 ± 3.24f, x (361)[n]	60.71 ± 3.09e, x (301)	46.67 ± 2.68d, x (228)	37.59 ± 2.75c, x (177)	28.87 ± 2.11b, x (142)	21.65 ± 2.57b, x (90)	10.47 ± 2.36a, x (41)
B	701	74.94 ± 2.84g, x (525)	61.27 ± 2.58f, x (442)	48.96 ± 2.49e, x (332)	37.12 ± 2.47d, x (250)	30.18 ± 2.11c, x (196)	20.37 ± 2.24b, x (127)	10.43 ± 1.97a, x (62)
B'	611	73.57 ± 3.18g, x (458)	60.11 ± 3.15f, x (359)	49.64 ± 3.07e, x (296)	38.24 ± 2.09d, x (234)	29.35 ± 1.39c, x (182)	18.70 ± 1.92b, x (120)	7.43 ± 1.84a, x (45)

a,b,c,d,e,f,g = Means within row with different letters are significantly different at p <0.05
x = Means within column with similar letters are not significantly different at p >0.05
[n]Number of embryos

oocytes, however, the mean percentage of developmental stages was not significantly different.

Discussion

The quality of cattle oocytes influenced the fertilization rate. All grades of oocytes were capable of forming pronuclear. The results were encouraging, in which higher fertilization rates were obtained in Grades A and B oocytes compared with the study by Leibfried-Rutledge et al. (1987). However, lower fertilization rate was obtained when compared with Grade B' oocytes.

Differences at the cytoplasmic level could explain the higher rate of development of oocytes in Grades A and B than those in Grade B'. Grade B' had the highest number of oocytes observed at metaphase II (MII) or with unidentified status following in vitro fertilization. Therefore, it is possible that due to the disconnection of junctional complexes, Grade B' oocytes could have attained incomplete cytoplasmic maturation and subsequently reduced their ability to mature and fertilize in vitro. This could explain the high incidence of polyspermy observed together with the high percentage of oocytes that were arrested at MII or with unidentified status in this grade of oocytes.

Cumulus cells secrete non-sulfated glycosaminoglycan hyaluronic acid around the oocyte that expands the spaces between the cells (Ball et al. 1982; Downs et al. 1986). Cumulus cells were also necessary at the time of fertilization to maximize the incidence of normal sperm acrosome reaction (Tanghe et al. 2003). Hyaluronic acid is effective for sperm preparation prior to IVF in cattle (Shamsuddin and Rodriquez-Martinez 1994) and improves developmental capacity of cattle embryos under in vitro conditions (Stojkovic et al. 2002).

In the present study, the fertilization medium used was supplemented with bovine serum albumin (BSA) as a protein source. Protein supplementation is essential to influence sperm penetration of cumulus-free oocytes but not in cumulus-intact oocytes (Tajik et al. 1993). It has been observed that the addition of protein during IVF seemed to play a significant role in enhancing pronuclei formation (Chen-Lu and Lu 1990; Tajik et al. 1994; Eckert and Niemann 1995). This might explain why Grade B' oocytes were still able to undergo fertilization, cleavage and subsequent blastocyst development.

In the present study, the BO medium which was supplemented with heparin and calcium ionophore might help in the pronuclei formation in all grades of oocytes, although lower fertilization rate was observed in poor quality grades of oocytes. Evidence suggests that heparin not only plays an important role in sperm capacitation and acrosome reaction but also improves pronuclear formation and early embryonic cleavage (Li et al. 2004). Either heparin alone or heparin combined with calcium ionophore significantly enhances the male pronucleus formation (Wei and Fukui 1999).

Other related studies also indicated that the presence of cumulus cells reduced the degree of zona pellucida 'hardening' that occurred during in vitro culture (Katska et al. 1989). Mochizuki et al. (1991) inferred that following oocyte maturation in the absence of cumulus cells and/or serum, the decrease in the fertilizability of oocytes was proportionate to the increase in zona-hardening, mainly due to the suppression of sperm penetration (Downs et al. 1986). Therefore, the low fertilizability of denuded oocytes might be due to the hardening of the zona pellucida and that the incomplete cytoplasmic maturation was due to the disconnection of junctional complexes.

Conclusion

The ability of oocytes to develop into embryos depended on normal nuclear and cytoplasmic oocyte maturation. Therefore, the presence of cumulus cells is essential for proper nuclear and cytoplasmic maturation of the oocytes and subsequently, pronuclear formation for blastocyst yield.

Acknowledgement

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Abstrak

Kesan kualiti oosit terhadap pembentukan pronukleus (PN) dan seterusnya perkembangan embrio lembu tempatan telah dikaji. Oosit lembu yang dimatang dan disenyawakan secara in vitro tidak berkeupayaan untuk terus berkembang tanpa sel kumulus. Kehadiran sel kumulus merangsang persenyawaan normal yang akan membentuk dua pronukleus (2PN). Ciri dan kualiti kompleks oosit-kumulus mempengaruhi pembentukan pronukleus tetapi tidak pada pembelahan dan pembentukan blastosista terkemudian. Oosit daripada Gred A, Gred B dan Gred B' berupaya untuk disenyawakan dan berkembang secara in vitro. Kadar persenyawaan antara ketiga-tiga gred adalah berbeza secara signifikan (p <0.05) dengan Gred A mempunyai kadar persenyawaan tertinggi (55.0%), diikuti oleh Gred B (44.1%) dan B' (30.2%). Polispermi (>2PN) pula berlaku pada kadar 18.8% dalam oosit Gred B' tetapi tidak dalam oosit Gred A dan B. Peratus purata kadar pembelahan dan kadar blastosista tidak berbeza secara signifikan (p <0.05) antara Gred A, B dan B' iaitu masing-masing, 71.6, 74.9 dan 73.8%, dan masing-masing 10.5, 10.4 dan 7.43%. Kesimpulan kajian ini menunjukkan kepadatan sel kumulus di sekeliling oosit mempengaruhi pembentukan pronukleus oosit lembu, manakala kadar pembelahan dan penghasilan blastosista tidak berubah.

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