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Asian Journal of Animal and Veterinary Advances

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Asian Journal of Animal and Veterinary Advances 6 (4): 322-332, 2011
ISSN 1683-9919 / DOI: 10.3923/ajava.2011.322.332

Relationships of Ca^{2+} Oscillations and ER Cluster Distribution to Porcine Oocyte Fragmentation

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ABSTRACT

Ca^{2+} is an important intracellular messenger during oocyte development. Ca^{2+} oscillations are dependent upon the Endoplasmic Reticulum (ER), an internal Ca^{2+} store capable of releasing and re-sequestering Ca^{2+}. However, mechanisms that regulate oocyte fragmentation are unclear. The aim of the current study was to investigate the mechanism of Ca^{2+} release and ER cluster distributions during fragmentation of aging culture porcine oocytes. After oocytes were cultured over a 40 h interval, ER cluster distributor and Ca^{2+} amplitudes significantly decreased. Oocyte fragmentation appeared after cells had been cultured 24 h and reached its peak at 48 h. At 40 h, normal morphology oocytes (NMor) had low Ca^{2+} amplitude and little ER cluster distributor; partial fragmentation (PFra) oocytes had less frequent and shorter amplitudes of Ca^{2+} release with ER cluster distribution partly aggregated in cytoplasm; whereas complete fragmentation oocytes (Cfra), which were increased by low voltage EA or enucleation, had no Ca^{2+} amplitude or ER cluster distribution. The number of PFra oocytes that proceeded to CFra following aging was increased in a Ca^{2+} free medium. We therefore conclude that extracellular Ca^{2+} abnormality resulted in altered intracellular Ca^{2+}, leading to changes in oocyte fragmentation dynamics.

Key words: Ca^{2+} oscillations, ER cluster distribution, oocytes fragmentation

INTRODUCTION

Ca^{2+} is one of the most important secondary messengers in cellular signaling in mammals (Berridge *et al*., 2000). Proper functioning of such signals depends upon the ability of cells to maintain low intracellular free Ca^{2+} concentration (Ca^{2+}) in resting conditions and to create rapid and transient elevation and subsequent re-uptake of Ca^{2+} upon appropriate stimulation. In response to extracellular stimuli, the intracellular free (Ca^{2+})-generated Ca^{2+} signal can regulate a diverse range of cellular responses (Berridge *et al*., 2000) and participate in numerous signal transduction pathways. Signal transduction during oocyte maturation and fertilization involves Ca^{2+} signals that are responsible for aging and death of oocytes (Cuthbertson *et al*., 1981). Nevertheless, the pattern of transient release of stored Ca^{2+} differs in oocytes undergoing maturation versus those that are aging (Hao *et al*., 2009).

Prolonged in vitro culture of oocytes is accompanied by complex processes described as aging. Among the variety of changes accompanying aging are altered intracellular Ca^{2+} homeostasis

(Igarashi *et al*., 1997) decreased activities of both MPF and MAP kinases (Kikuchi *et al*., 1999, 2000) and increased risk of polyspermy. Furthermore, these changes can result in increased fragmentation frequency in aged porcine oocytes (Petrova *et al*., 2004). The extent to which changes in Ca^{2+} concentrations are involved in fragmentation of aged oocytes is not known.

Mammalian oocytes maintain full developmental competence up to the optimal time after ovulation for fertilization, but if oocytes are not then fertilized, aging oocytes will undergo spontaneous fragmentation (Perez *et al*., 1999). Following delayed fertilization, oocytes can begin development, but low developmental competence and a high rate of fragmentation have been observed (Tarin *et al*., 1999; Tanabe *et al*., 1982). Following such delayed fertilization, fragmentation may be induced by initiation of the (Ca^{2+}) signal, which activates caspases in aged oocytes, implying that the (Ca^{2+}) signal may act as a trigger of programmed cell death in aged oocytes (Gordo *et al*., 2000).

Interestingly, Ca^{2+} responses in aged mouse oocytes are abnormal in several respects (Jones and Whittingham, 1996; Igarashi *et al*., 1997). For instance, the rate of increase of (Ca^{2+}) during fertilization or after stimulation by agonists is slower in aged than in younger oocytes. Likewise, the amplitude of the responses and the duration of the oscillations are decreased in aged oocytes. Collectively, these results indicate that Ca^{2+} homeostasis is altered in aged oocytes (Takahashi *et al*., 2000). Whether, (Ca^{2+}) is altered in *in vitro*-aged porcine oocytes is not known. In light of these observations, we investigate whether the characteristics of Ca^{2+} release induced by Electro-Activation (EA) and ER cluster distributer are associated with fragmentation in aged oocytes. Furthermore, we examine whether extracellular Ca^{2+} and ER cluster distributer dynamics result in the alteration of Ca^{2+} homeostasis, resulting in fragmentation in aged oocytes.

The present study investigates the following changes in porcine oocytes after different durations of culture: (1) Fragmentation morphology and rate as well as endoplasmic reticular distribution; (2) Ca^{2+} release patterns of oocytes having different fragmentation morphology after EA; (3) Fragmentation morphology and developmental competence in a Ca^{2+} free medium; (4) Fragmentation of enucleated oocytes.

MATERIALS AND METHODS

This research project belong to Ph.D Project of China Agriculture university. It was belong to China National 863 Program with grant No. (12008AA101003). Project was conducted from Jan. 2008 to Dec. 2009 in China Agricultural University in Beijing, we also do few Ca^{2+} release experiment in Beijing University.

Oocyte collection and *in vitro* maturation (IVM): All chemicals used in this study were purchased from Sigma Chemicals (St. Louis, MO, USA) unless specified otherwise.

***In vitro* maturation (IVM) of oocytes:** Ovaries from white crossbred gilts were collected at a local slaughterhouse and transported to the laboratory in 30-35°C 0.9% NaCl with 75 mg L^{-1} penicillin and 50 mg L^{-1} streptomycin within 2 h after slaughter. Follicles 2-5 mm in diameter were aspirated using an 18 G needle and syringe. Cumulus Oocyte Complexes (COCs) with several layers of unexpanded cumulus cells were selected, rinsed three times in the maturation medium and then incubated at 39°C for 44 h with 100% humidity and 5% CO_2 in air (Rahman *et al*., 2007). The maturation medium was a defined medium consisting of Tissue Culture Medium (TCM) 199 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% porcine follicular fluid, 10 IU mL^{-1} hCG

(Chorulon, Intervet Australia Pty Limited, Victoria, Australia), 10 IU mL^{-1} eCG (Folligon, Intervet Australia Pty Limited, Victoria, Australia), 10 ng mL^{-1} EGF, 0.60 mM cysteine, 75 mg L^{-1} penicillin and 50 mg L^{-1} streptomycin.

***In vitro* aging of oocytes:** After maturation, cumulus cells were removed by gentle pipetting in the presence of 0.1% hyaluronidase. Oocytes were then cultured in PZM-3 or PZM-3 Ca^{2+} free ($Ca\text{-}(lactate)_2 \cdot 5H_2O$ was replaced by $Na\text{-}lactate \cdot 5H_2O$) droplets (Yoshioka *et al.*, 2002) (15) for *in vitro* aging, covered with mineral oil at 39°C, 5% CO_2 in humidified air. At 12 h intervals, oocytes were randomly chosen for evaluation of fragmentation and self-activation, to be described.

Parthenote production and culture: Oocytes were transferred to activation medium containing 0.3 mannitol, 1.0 mM $CaCl_2$, 0.1 mM $MgCl_2$ and 0.5 mM HEPES. Electro-Activation (EA) was induced by one DC pulse of 130 V mm for 80 ìs with a cell fusion machine (Fujihira Industry Co. Ltd., Japan). Oocytes were then cultured in 2 mmol L^{-1} 6-dymethylaminopurine (6-DAMP) for 6 h. Parthenotes were cultured in PZM-3 (Yoshioka *et al.*, 2002) at 39°C, 5% CO_2 in humidified air for 7 days. Cleavage embryos were observed on day 2 and blastocyst formation was observed on day 7. Experiments were repeated three times, with greater than 30 oocytes per repeat.

Hoechst staining: By morphology, oocytes with visible protuberances and blastomeres were selected. After being washed three times, oocytes were incubated with PBS containing 10 μg mL^{-1} Hoechst 33342 at room temperature in the dark for 10 min. Oocytes were then mounted on a slide and covered with a cover slip. Fluorescence intensity was determined with a fluorescence microscope (Olympus, BX60, Japan). Images were recorded with a digital camera (Nikon 990, Tokyo, Japan).

Observation of endoplasmic reticulum in oocytes by confocal microscopy: Endoplasmic reticular samples were dyed using General ER Staining Kit (GMS10041.1v.A, GENMED SCIENTIFICS INC. USA) according to the manufacturer's instructions. Thirty intact oocytes selected randomly from each group were washed three times in M199 and fixed in 4% paraformaldehyde solution at 4°C for 1 h. After fixation, oocytes were washed three times in TCM 199 containing 25 mm HEPES and then incubated in reagent A supplemented with ER fluorescence probe solution (3,3-Dihexyloxacarbovyanine iodide) which is carbocyanine positive ion fluorescence dye at room temperature for 1 min. Following three more washes in TCM 199 containing 25 mm HEPES (H199), oocytes were mounted on a slide, covered with a cover slip and examined under laser scanning confocal microscopy (Nikon, TE-2000-E, Tokyo, Japan). The ER fluorescence dye was excited with 488 nm light and emitted fluorescence was collected with a 505 nm long pass filter. Experiments were repeated three times.

Detection of intracellular free calcium ion in oocytes after electro-activation: Calcium content in oocytes after EA was measured according the method of Sun *et al.* (1992) with some minor modifications. Oocytes at different stages were incubated in TCM 199 supplemented with 4 μm fura-2/AM (Invitrogen) at 38.5°C for 50 min. After three washes in M199, fura-2/AM-loaded oocytes were transferred into TCM 199 containing 25 mm HEPES and stimulated by a single DC electrical pulse of 1.3 kV cm for 80 μs at room temperature (22-23°C). The free calcium content in ooplasm was calculated by measuring the ratio of fura-2 fluorescence intensity at 530 nm excited by ultraviolet light at 340 and 380 nm, respectively. Experiments were repeated three times, with greater than 30 oocytes per repeat.

Enucleation by micromanipulation: Oocytes were enucleated by micromanipulation using a Nikon Diaphot TMD inverted microscope fitted with Nomarski differential interference contrast optics and a Narishegi micromanipulation system (Nikon). Oocytes were placed into 20-pl microdrops of TCM 199 HEPES containing 5.0 pg mL^{-1} cytochalasin B and were covered with mineral oil (Sigma). The first polar body and approximately one third to one half of the oocyte cytoplasm was aspirated through use of a micropipette with an internal diameter of 25 m. After micromanipulation, oocytes were placed in TCM 199 supplemented with 10% FCS for 6 h at 39°C in 5% CO_2 in air. The karyoplasts removed by micromanipulation were individually stained with Hoechst 33342 and then checked for the presence of metaphase II chromosomes by epifluorescent microscopy to confirm whether the corresponding oocyte had been successfully enucleated.

Statistical analysis: All data were subjected to analysis of variance using the GLM procedures of Statistical Analysis System (SAS Institute, Cary, NC, USA). Differences among oocytes from different stages of culture were determined by using Duncan's multiple-range test. All data were expressed as Mean±SEM. The percentages were subjected to arcsine transformation prior to analysis. Differences were considered to be significant when $p<0.05$.

RESULTS

Experiment 1: Fragmentation morphology of aging oocytes in PZM-3.

Observation of the morphology of aging oocytes in vitro cultured in PZM-3: After *in vitro* maturation (as 0 h), denuded oocytes had uniformly distributed cytoplasm of similar appearance as well as intact plasma membranes (Fig. 1, A1). After 24 h of *in vitro* aging, some oocytes showed initial condensation of cytoplasm (retraction of the oolemma from the zona pellucida) and a few oocytes were fragmented. After 36 h of aging, oocytes presented three different morphologies: Normal (NMor (Fig. 1, B1)), Partial fragmentation (PFra (Fig. 1, C1)), which included a few oocytes (0.07±0.03) caused by self-activation of calcium and Complete fragmentation (CFra) (Fig. 1, D1).

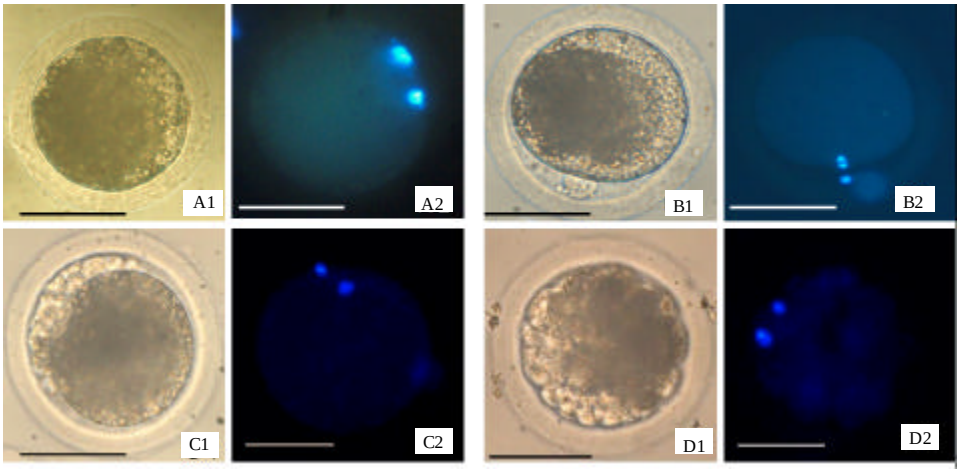

Fig. 1: Morphology of porcine *in vitro*-aged oocytes. (A1, A2) An oocyte maturation cultured for 44 h forms a nucleus and a polar body. (B1, B2) Aged 36 h *in vitro*, this NMor oocyte has a nucleus and a polar body. (C1, C2) Aged 36 h *in vitro*, this PFra oocyte has a nuclei and a polar body. (D1, D2) Aged 36 h *in vitro*, this CFra oocyte has a nucleus and a polar body. Bar = 30 μm

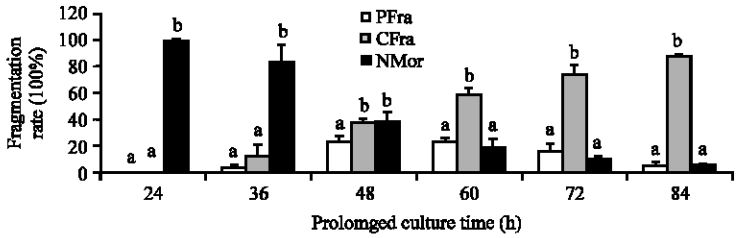

Fig. 2: The rate of different fragmentation morphology porcine at culture different time in PZM-3. The CFra and PFra increased along with aging. CFra rate significantly increased from 24 h, some of PFra oocytes keeped fragmtating and finally became CFra. The PFra rate of oocytes was significantly lower than CFra ($p<0.05$; 48 h (22.8±5.1 vs. 37.7±2.1), 60 h (22.7±3.6 vs. 58.9±4.1), 72 h (15.9±6.4 vs. 73.6±8.2), 84 h (5.9±1.7 vs. 87.8±1.4). Different superscripts above each group bar represent significant difference ($p<0.05$)

Additional aging beyond 36 h, the oocytes of NMor had almost disappeared, whereas CFra and PFra oocytes notably increased and many PFra oocytes had progresses to CFra oocytes. Most fragmented oocytes had a nucleus and a polar body (Fig.1, C2, D2).

The fragmentation rate of aging porcine oocytes having different morphologies in PZM-3: The fragmentation of oocytes increased with aging (Fig. 2), as oocytes with normal morphology transitioned into the partially fragmented class which subsequently transitioned to complete fragmentation.

Experiment 2: The ER cluster distribution in aging porcine oocytes after culture for different durations.

Most of the oocytes that were cultured in PZM-3 for 0 h had abundant ER cluster distribution (88.76%±0.9, 87/98) (Fig. 3a). ER distribution of NMor oocytes cultured for 36 h (72.73%±4.6, 64/88) exhibited weak staining (Fig. 3b). The ER distribution of Pfra oocytes was aggregated (Fig. 3c) in a portion of the cytoplasm (75.56%±3.8, 68/90). No ER distribution was observed in CFra oocytes (80.85%±5.1,76/94) (Fig. 3d).

Experiment 3: Calcium release patterns induced by electro stimulation in porcine aged oocytes. Ca^{2+} transient release is a reflection of ability of ER Ca^{2+} release. One monotonic electrical stimulation generated one monotonic increase in ooplasmic free calcium, but calcium concentration rise to plateau of fragmentation groups became shorter than control with prolonged culture time *in vitro* (Table 1). The amplitude of (Ca^{2+}) rise after EA were significantly higher in control (Fig. 4a) than in NMor (Fig. 4b), PFra (Fig. 4c) and CFra (Fig. 4d) group. The amplitude of (Ca^{2+}) rise after EA was significantly higher in NMor group than in PFra and CFra groups ($p<0.05$) and the amplitudes of (Ca^{2+}) were demonstrably higher in the Pfra group than in the Cfra group ($p<0.05$).

Experiment 4: Effect of calcium and calcium-related factors on fragmentation of porcine oocytes.

Experiment 4.1: Effect of Ca^{2+} Free PZM-3 on fragmentation morphology and fragmentation rate of porcine oocytes.

The effect of Ca^{2+} in the medium on oocyte fragmentation was investigated by comparing oocytes from prolonged culture in Ca^{2+} Free PZM-3 versus control medium. The rate of PFra oocytes

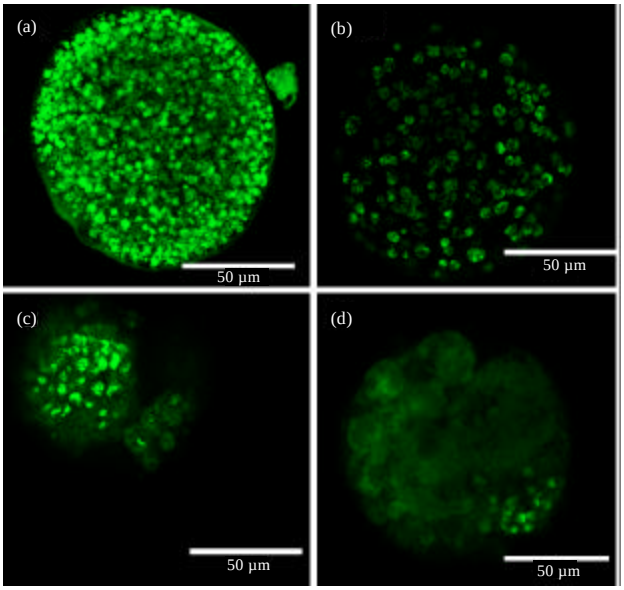

Fig. 3: ER cluster distribution of fragmentation porcine oocytes. (a) Control, porcine oocytes cultured in PZM-3 for 0 h, had abundant ER cluster distribution. (b) ER cluster distribution of NMor oocytes is sharply decreased at aged 40 h, (c) ER cluster distribution of PFra oocytes is partly aggregated in cytoplasm at aged 40 h. (d) ER cluster distribution is absent in CFra oocytes at aged 40 h. Bar = 50 µm

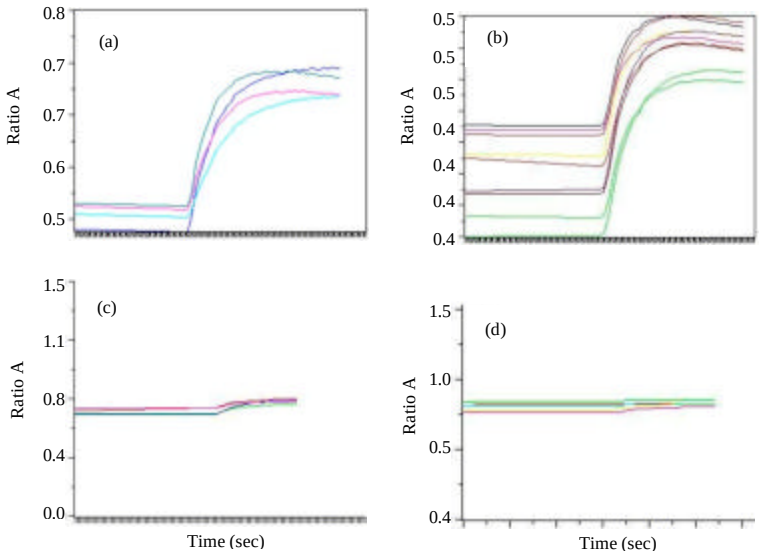

Fig. 4: Calcium release pattern of fragmentation oocytes at aging 40 h induced by electro-stimulation. (a) Calcium release pattern of control (MII oocytes, which were maturation for 44 h or aged 0 h). (b) calcium release pattern of Nmor oocytes at aged 40 h. (c) Calcium release pattern of PFra oocytes at aged 40 h. (d) Calcium release pattern of CFra oocytes at aged 40 h. Each line denotes one oocyte. Ratio = fluorescence 340 nm: fluorescence 380 nm

Table 1: ER calcium amplitude of porcine oocytes after aged 40 h

Groups	No. of oocytes examined	Amplitude of (Ca^{2+})
Control	114	0.211±0.053[a]
NMor	90	0.084±0.009[b]
PFra	108	0.044±0.006[c]
CFra	134	0.008±0.008[d]

*Values in the same column with no common superscripts differ significantly ($p<0.05$)

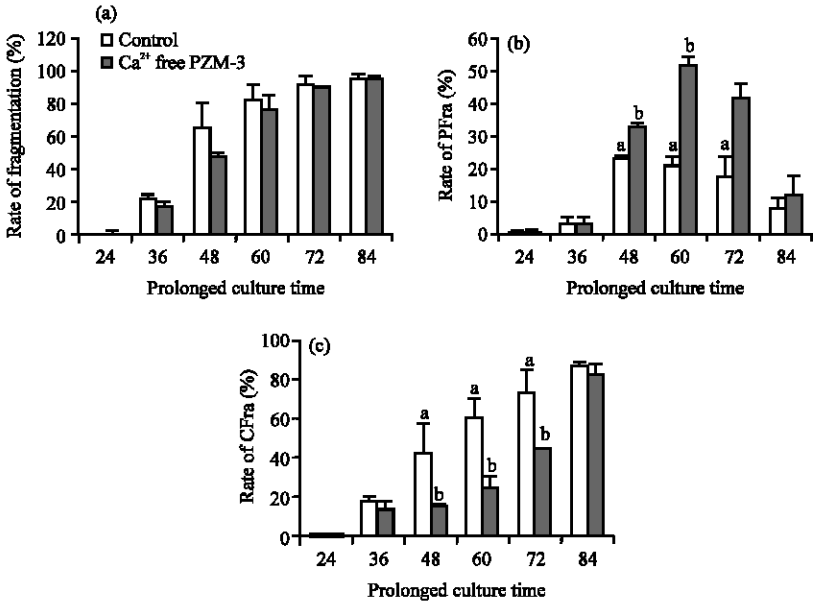

Fig. 5: Different fragmentation morphology of porcine oocytes in Ca^{2+} Free PZM-3.The PFra rate was significant higher in Ca^{2+} Free PZM-3 than in control media ($p<0.05$) at 48 , 60 and 72 h. Bars represent Mean±SEM. Different superscripts above each group bar represent significant difference ($p<0.05$)

in Ca^{2+} Free PZM-3 gradually increased (Fig. 5b) and the PFra rates were significantly higher (Fig. 5b) than control ($p<0.05$) at 48 h (32.93±1.08 vs. 22.70±0.76), 60 h (51.13±3.38 vs. 21.03±2.70) and 72 h (41.77±4.41 vs. 17.35±6.37), but there were no differences at 128 h ($p>0.05$). At the same time, the proportion of CFra oocytes in Ca^{2+} Free PZM-3 was significantly lower than control ($p<0.05$) at 48 h (15.19±0.92 vs. 43.06±13.82), 60 h (24.95±5.7 vs. 61.39±8.29) and 72 h (44.96±0.99 vs. 74.28±13.12).

Experiment 4.2: Effect of low voltage EA on fragmentation morphology and fragmentation rate of porcine oocytes

In order to determine whether extracellular Ca^{2+} affected oocyte fragmentation, oocytes were observed following low voltage stimulation (1.6 kv mm for 80 μs) (Fig. 6a, b). The proportion that were fragmented was not significantly increased over the control during prolonged culture (Fig. 6b), but the CFra oocytes significantly increased (Fig. 6a) over the control at 48 h (70.79±10.29 vs. 41.59±0.38), 60 h (92.80±5.05 vs. 53.37±2.01) and 72 h (94.88±2.33 vs. 80.05±3.01).

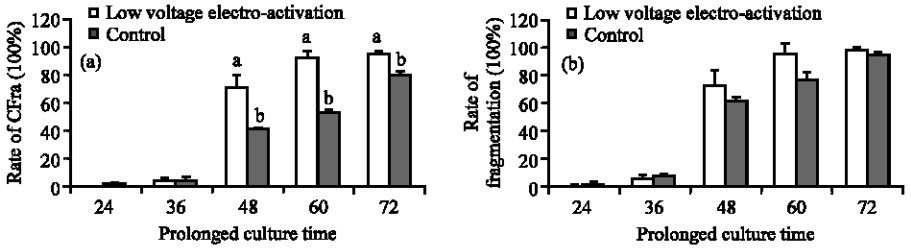

Fig. 6: Effect of low voltage EA on fragmentation morphology of porcine oocytes. The CFra rate was significantly increased by low voltage EA at 48, 60 and 72 h, compared to the control ($p<0.05$) and there was virtually no cleavage of oocytes in the low voltage EA group. Bars represent Mean±SEM. Different superscripts above each group bar represent significant difference ($p<0.05$)

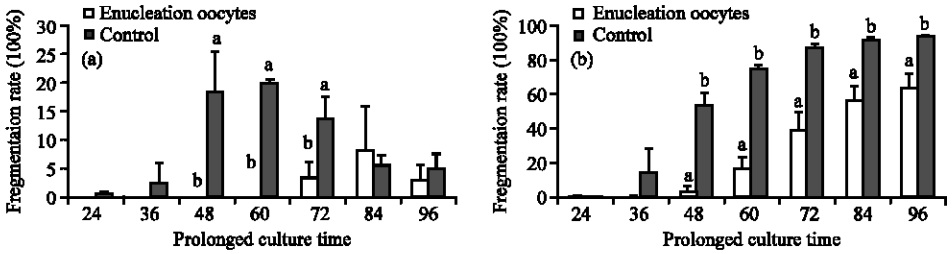

Fig. 7: Fragmentation morphology of enucleate porcine oocytes in PZM-3. Fragmentation proportions were significantly decreased from control at 48, 60, 72 h and 84 h ($p<0.05$). The PFra rate was significantly decreased at 48, 60 and 72 h compared to control ($p<0.05$), Bars represent Mean±SEM. Different superscripts above each group bar represent significant difference ($p<0.05$)

Experiment 5: Effect of enucleate factors on fragmentation of porcine oocytes

In order to determine whether PFra oocytes begin fragmentation at the site of the polar body, enucleated oocytes were cultured for a prolonged period in PZM-3. Rate of fragmentation and PFra were significantly decreased compared to control oocytes culture (Fig. 7). Fragmentation rate was significantly lower (Fig. 7b) than control at 48 h (3.76±1.00 vs. 53.97±1.00), 60 h (17.31±1.00 vs. 75.3±14.81), 72 h (39.76±3.54 vs. 87.53±2.45), 84 h (56.42±10.99 vs. 92.38±1.47) and 96 h (63.66±9.52 vs. 93.77±0.74). PFra rate was significantly lower than control (Fig. 7a) at 48 h (0±0 vs. 18.54±7.11), 60 h (0±0 vs. 19.78±0.70) and 72 h (3.09±3.09 vs. 13.63±3.99).

DISCUSSION

It is well known that (Ca^{2+}) oscillations are responsible for promoting progression of the cell cycle in mammalian oocytes and that Ca^{2+} release may also be responsible for changes in aged and fragmentation oocytes, at least in part. Perez *et al.* (1999) and Petrova *et al.* (2004) have demonstrated oocytes fragmentation and Kim *et al.* (2008) also proved the ER stress is related with fragmentation. However, the fragmentation and change of ER cluster distribution occur almost simultaneously and may be linked by common mechanisms. The present study suggests that ER cluster distribution and amplitude of (Ca^{2+}) dramatically decreased in aging fragmentation porcine

oocytes, Which is consisted with mention of Nicotera *et al.* (1992). Altered Ca^{2+} homeostasis after EA may be an important factor in inducing fragmentation in aged oocytes. Although, the mechanisms regulating oocyte aging and fragmentation are poorly defined, they may involve decrease of ATP in conjunction with reduced Bcl-2 expression in aged oocytes (Gordo *et al.*, 2002). Research from our laboratory has shown that prolonged culture results in progressive decrease in ATP content (Hao *et al.*, 2009). Low concentration of ATP may impair the function of Ca^{2+}-ATPase pumps, which are responsible for Ca^{2+} re-uptake of ER. Reduced amounts of Bcl-2 may also negatively impact the function of the Ca^{2+} pump. Malfunction of the Ca^{2+} pump may therefore be responsible for abnormal Ca^{2+} homeostasis in aged oocytes.

In the present study, ER cluster distribution was related to amplitude of (Ca^{2+}) in NMor, PFra and Fra oocytes. Many studies have shown that there is specialized communication between ER and Ca^{2+} release (Csordás *et al.*, 1999; Szalai *et al.*, 1999). The ER (Ca^{2+}) values need to be sustained at suitable levels and (Ca^{2+}) with the equilibrium regulated by Ca^{2+}-ATPases between the cytosol and ER. Unless the ER Ca^{2+} uptake is prevented or the ER system experiences high transmembrane leaks, steady state values of ER (Ca^{2+}) would be maintained. In response to pathological stimuli associated with aging, the initiation of (Ca^{2+}) oscillations may result in depletion of Ca^{2+} from the ER, but (Ca^{2+}) re-uptake ability is inadequate, as evidenced by Ca^{2+} oscillations of aged oocytes at different periods. Low Ca^{2+} in the ER is an effective trigger of cell death in somatic cells (Jiang *et al.*, 1994; Bian *et al.*, 1997) and also suppresses protein synthesis (Soboloff and Berger, 2002), either of which could severely limit embryo development. The ER cluster distribution was scanty and heterogeneous in fragmented oocytes, indicating appreciable heterogeneous kinetic constraints to intracellular diffusion of Ca^{2+} (Tsien and Tsien, 1990). Existence of similar processes could account for the low ER (Ca^{2+}) values in intracellular compartments, such as abnormal ER cluster distributor inefficiently programmed for the uptake and release of calcium. Otherwise, prolonged culture results in decrease in ATP content and prevention of mitochondrial clustering during oocyte aging (Hao *et al.*, 2009). Based on the above considerations, change of ER cluster distribution might promote oocyte fragmentation.

In the present study, the proportion of PFra oocytes was increased in Ca^{2+} free medium, following which CFra was increased by low voltage EA. Our results demonstrate that intracellular (Ca^{2+}) concentration affected the rate of oocyte fragmentation and that extracellular Ca^{2+} was needed to stimulate oocyte fragmentation, Kang and Park (2005) have demonstrated extracellular Ca^{2+} related with the intracellular Ca^{2+}. Change of extracellular Ca^{2+} concentration could produce membrane hyperpolarization and a concentration-dependent change of intracellular Ca^{2+} due to Ca^{2+} influx across the plasma membrane. Besides, it was transported back to the ER and to other organelles such as mitochondria. Therefore, changes of extracellular Ca^{2+} concentration certainly could affect intracellular Ca^{2+} homeostasis, which in turn could modulate oocyte fragmentation.

In the present experiments, enucleated oocytes had an extremely low rate of PFra and fragmentation was delayed. This indicates that nucleus and nuclear envelope were important for fragmentation of oocytes. It is now recognized that the nuclear envelope itself functions as a Ca^{2+} storage pool (Maruyama *et al.*, 1995) and has receptors for InsP3, InsP4 and ADP-ribose. The Ca^{2+} response of enucleated oocytes promoted by InsP3 was significantly slowed (Lim *et al.*, 2001), resulting in low cytoplasmic Ca^{2+} concentrations (Tanabe *et al.*,1982). Otherwise, the apoptosis signal induced by DNA fragmentation was inhibited due to removal of nuclei. All these reasons could contribute to delaying oocyte fragmentation.

CONCLUSION

The ER distribution changed from coarctate to scanty and amplitude of (Ca^{2+}) decreased as culture time of porcine oocytes increased. Along with diversity of ER distributer and reduced capacity for Ca^{2+} release, intracellular Ca^{2+} homeostasis was disturbed and oocyte fragmentation was accelerated. In addition, extracellular Ca^{2+} might evoke changes in intracellular Ca^{2+}, accordingly resulting in the change of fragmentation morphology. Fragmentation of enucleated oocytes also was delayed, which might be due to the same mechanism.

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