Journal of Animal and Veterinary Advances 11 (14): 2516-2522, 2012
ISSN: 1680-5593

Spatiotemporal Dynamincs and Concentrations of Intracellular Free-Ca^{2+} in *in vitro* Matured and *in vitro* Fertilized Bovine Oocytes and Very Early Embryo Stages

Lu Li, Dong Zhang, Junhai Lian and Huanmin Zhou
College of Life Science, Inner Mongolia Agricultural University, 010018 Hohhot, China

Abstract: This study aimed to measure the concentration of intracytoplasmic free-Ca^{2+} in the *in vitro* matured bovine oocytes at MII stage and in the *in vitro* fertilized embryos at early different developmental stages 0 h (fertilization), 2 and 15 h (pronucleus stage) and 48 h (2-cell stage) after fertilization using Fura-2/AM as a Ca^{2+} fluorescent probe and to investigate Ca^{2+} spatial distribution in the intracytoplasm of bovine eggs at MII stage and 0, 2, 15 and 48 h after fertilization using Ca^{2+} fluorescent probe Fluo-3/AM. Meanwhile, the fertilized eggs were cultured in Ca^{2+}-free culture medium to examine the effects of extracellular Ca^{2+} on the early embryo development. The results indicated that the intracellular Ca^{2+} concentrations of the bovine oocytes at MII stage and 0, 2, 15 and 48 h after fertilization were 84.99 nmol L^{-1} and 120.05, 229.09, 108.73 and 111.17 nmol L^{-1}, respectively. No pronuleus or cleft eggs were found when cultured in Ca^{2+}-free culture medium. These results suggested that the fertilization induced intracytoplasmic free-Ca^{2+} increase that pronucleus formation and egg cleavage were Ca^{2+} dependent that extracellular Ca^{2+} performed a specific function for the initiation of zygote development and that comparatively, a high level of Ca^{2+} concentrations was still maintained after pronucleus formation. Moreover, the spatial profile of fluorescence expression illustrated that a low density free-Ca^{2+} were homogenously distributed in the cytoplasm of oocytes at MII stage, the Ca^{2+} were concentrated at the cortex zone under the egg plasma membrane at fertilization, the Ca^{2+} intensity enhanced and spread towards the center of egg from edge at 2 h after fertilization and the Ca^{2+} were distributed in whole cytoplasm at pronucleus and first cleavage stage. These results demonstrated that extracellular Ca^{2+} is essential for normal embryonic development and also, confirm that Ca^{2+} increase is required for bovine oocyte activation at fertilization.

Key words: Bovine, oocyte, calcium, *in vitro* fertilization, IVF embryo, China

INTRODUCTION

For most mammalian species, the oocytes arrested at metaphase of the second Meiotic division (MII) before fertilization. Withdrawal from the MII arrest may depend on the response of the oocyte to extracellular or/and intracellular signals. It has been known that there is a transient rise in the cytosolic free-Ca^{2+} concentration that acts as the trigger for the events associated with oocyte meiotic resumption (Deguchi and Osanai, 1994; Malcuit *et al*., 2006; Jones, 2005). An increase induced by sperm in the egg intracellular Ca^{2+} concentration was first observed in medaka eggs over two decades ago (Ridgway *et al*., 1977). Such an increase has since been found in a variety of animal species and even during fertilization of species in the plant kingdom (Digonnet *et al*., 1997). The mammalian oocytes exhibit repetitive transient increase termed as oscillation in the cytosolic free-Ca^{2+} at the time when the meiotic resumption of oocytes is initiated for fertilization (Deguchi and Osanai, 1994; Jones *et al*., 1995a; Malcuit *et al*., 2006). These Ca^{2+} oscillations continue past through polar body extrusion and cease at pronucleus formation in the newly formed zygote (Ducibella *et al*., 2002; Jones *et al*., 1995b). Moreover, a possible Ca^{2+} signal transduction pathway for the meiotic resumption of mammalian oocytes at fertilization has been postulated though its molecular mechanism has not been fully understood. The Ca^{2+} release, derived primarily from the egg's endoplasmic reticulum (Kline and Kline, 1992) is triggered by the interaction of sperm-egg.

The initial wave of Ca^{2+} propagates from the point of sperm-egg fusion and generates a long-lasting series of Ca^{2+} oscillations (Mehlmann *et al*., 1995, 1996; Shiraishi *et al*., 1995). These observations have been documented in murine (Miyazaki *et al*., 1986; Cuthbertson and Cobbold, 1985; Ben-Yosef *et al*., 1993) bovine (Fissore *et al*., 1992), porcine (Sun *et al*., 1992) and human (Taylor *et al*., 1993). However, most of the research focused on the pattern of Ca^{2+} oscillations at fertilization. Little information is available on real-time measurement of intracytoplasmic free-Ca^{2+} concentrations and especially,

Corresponding Author: Huanmin Zhou, College of Life Science, Inner Mongolia Agricultural University, 010018 Hohhot, China

spatial distributions of Ca^{2+} at different stages of fertilization for *in vitro* fertilized bovine eggs. The specific objectives of this study were:

- To measure the concentrations of intracytoplasmic free Ca^{2+} of bovine oocyte at MII and 0, 2, 15 and 48 h after fertilization in Ca^{2+} culture medium and 2 h in Ca^{2+}-free culture medium
- To examine the effects of extracellular Ca^{2+} on the development of the zygotes
- Further to investigate the correlation between early development and the concentration dynamics of Ca^{2+} in bovine oocytes and early embryos

MATERIALS AND METHODS

All chemicals were purchased from Sigma-Aldrich Company (St Louis, USA) unless state otherwise.

Collection and *in vitro* maturation of oocyte: Bovine ovaries were collected from local slaughterhouse and transported to the laboratory within 4 h at 25-30°C in physiological saline with penicillin (200 μg mL^{-1}) and streptomycin (200 μg mL^{-1}). Cumulus Oocyte Complexes (COCs) and follicular fluid were aspirated from follicles of 2-6 mm in diameter using a 12 gauge needle equipped to a 20 mL disposable syringe and transferred to a 90 mm culture dish. The COCs were selected under a stereomicroscope (Olympus, Tokyo, Japan). The oocytes morphologically characterized by homogenous cytoplasm and enclosed within at least three layers of viable compact cumulus cells were deemed suitable for *in vitro* maturation.

The selected COCs were washed three times in maturation medium, composed of 25 mM mL^{-1} HEPES-buffered TCM-199 (Gibco, BRL, USA) added with 10% Fetal Bovine Serum (FBS), 1 mM mL^{-1} sodium pyruvate, 26.19 mM mL^{-1} $NaHCO_3$, 60 μg mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, 20 μg mL^{-1} LH (Ningbo Hormone Factory, China), 10 μg mL^{-1} FSH (Ningbo Hormone Factory, China) and 1.5 μg mL^{-1} 17β-estradiol). Four 50 μL^{-1} microdrops of maturation medium were made under mineral oil in a 35 mm petri dish and pre-balanced in a CO_2 incubator (38.5°C, 5% CO_2 in air with 100% humidity) for at least 2 h. A group of 10-15 COCs were randomly assigned to each droplet then cultured in CO_2 incubator for 22 h for maturation.

At the end of maturation, the matured eggs were cultured in HEPES-buffered TCM-199 with 0.2% hyaluronidase in CO_2 incubator for 5 min and denuded all of cumulus cells by gently repeated pipetting. Only the morphologically normal eggs were selected for the measurements of intracytoplasmic free-Ca^{2+} concentration.

Fertilization of bovine eggs and embryo culture: Bovine frozen semen were thawed in a 37°C water bath for 30 sec. The thawed sperms were washed twice, 5 min for each time through, centrifugation (350 g) in Brackett and Oliphant (BO) medium (114.56 mM mL^{-1} NaCl, 4.03 mM mL^{-1} KCl, 2.25 mM mL^{-1} $CaCl_2$, 0.818 mM mL^{-1} $NaH_2PO_4 \cdot 2H_2O$, 0.805 mM mL^{-1} $MgCl_2 \cdot 6H_2O$, 46.82 mM mL^{-1} $NaHCO_3$, 0.33 mM mL^{-1} sodium pyruvate, 13.89 mM mL^{-1} glucose, 50 μg mL^{-1} penicillin and 60 μg mL^{-1} streptomycin). The live sperms that precipitated in centrifugal tube were collected and diluted by 1 fold with fertilization medium, comprised BO medium supplemented with 8 μL mL^{-1} heparin and 20 μg mL^{-1} Bovine Serum Albumin (BSA). Soon afterwards, the sperms were cultivated in fertilization medium containing 10 mM mL^{-1} caffeine for 5 h for capacitation in CO_2 incubator (38.5°C, 5% CO_2 in air with 100% humidity). The final concentration of sperms was adjusted to 2×10^6 mL^{-1}. A batch of 20-25 IVM eggs were seeded in a 100 μL droplet of sperm-contained fertilization medium which were pre-balanced for a half of hour and covered with mineral oil and then incubated in CO_2 incubator (38.5°C, 5% CO_2 in air with 100% humidity) for fertilization. Four droplets were made in each 35 mm petri dish. The extrusion of the second polar body was recognized as criteria for evaluating the fertilization of eggs.

After fertilization, the fertilized eggs were collected and divided into two groups. One group was continuously cultured in Ca^{2+} Synthetic Oviduct Fluid (SOF) supplemented with 20 μg mL^{-1} BSA and another in Ca^{2+}-free SOF supplemented with 20 μg mL^{-1} BSA for 48 h. The fertilized eggs cultured in Ca^{2+} medium were collected each hour during culture and stained with 1% orcein to assess their developmental stages. Based on the assessment, the fertilized eggs in Ca^{2+}-free medium were examined for developmental status at 15 (pronuclear stage) and 48 h (2-cell stage) of culture.

At 0, 2, 15 and 48 h of culture in Ca^{2+} medium, the fertilized eggs were cultured in HEPES-buffered TCM-199 with 0.2% hyaluronidase in CO_2 incubator for 5 min and denuded all of cumulus cells by gently repeated pipetting. The morphologically normal zygotes were selected for the measurements of intracytoplasmic free-Ca^{2+} concentration.

Measurement of intracytoplasmic free-Ca^{2+}: The eggs and the embryos at the different developmental stages were loaded with the calcium-sensitive fluorescent dye, fura-2 acetoxymethyl ester (fura-2/AM) by culture in well-equilibrated drops of 5 μM L^{-1} Fura-2/AM in working medium (94.66 nM mL^{-1} NaCl, 4.78 nM mL^{-1} KCl, 1.71 mM mL^{-1} $CaCl_2 \cdot H_2O$, 1.19 mM mL^{-1} KH_2PO_4 1.19 mM mL^{-1} $MgSO_4 \cdot 7H_2O$, 4.15 mM mL^{-1}

$NaHCO_3$, 20.85 mM mL^{-1} HEPES, 23.28 mM mL^{-1} sodium lactate, 0.33 mM mL^{-1} sodium pyruvate, 5.56 mM mL^{-1} glucose, 4 mg mL^{-1} BSA, 0.25% Pluronic-127, 50 μg mL^{-1} penicillin and 60 μg mL^{-1} streptomycin) in CO_2 incubator for 40 min. Finally, the Fura-2/AM-loaded eggs and embryos were rinsed in the working medium free of dye and maintained in Hank's medium at 38.5°C till use for measurement of Ca^{2+} contents.

Measurements of intracytoplasmic free-Ca^{2+} concentration were performed on a fluorescent photometer (Hitachi, Japan) using a modified approach (Deng *et al*., 1995; Dildy and Leslie, 1989; Sheu and Sharma, 1999). Briefly, the wavelength of emission light was 500 nm and the wavelength of excitation was 300-420 nm while the excitation wavelength for Fura-2/Ca^{2+} was 340 nm. Since, the fluorescent intensity of Fura-2/Ca^{2+} was in direct proportion to Ca^{2+} content, intracellular free Ca^{2+} concentration can be estimated as formula as following equation:

$$[Ca^{2+}] = K_d \times (F - F_{min})/(F_{max} - F)$$

Where:

K_d = The dissociation constant of Fura-2/Ca^{2+} (K_d = 224 nM L^{-1})

F = The value of fluorescent intensity of the oocyte or embryo suspension at peak excitation wavelength

F_{max} = The maximum value of fluorescent intensity of the oocyte or embryo suspension added with Triton X-100 at 0.1% final concentration

F_{min} = The minimum value of fluorescent intensity of the oocyte or embryo suspension added with Ethylene Glycol bis (2-aminoethyl) Ether-N, N, N, N-Tetraacetic Acid (EGTA) at 5 nM L^{-1} concentration on the base of measurements for F_{max}

All the measurements were carried out at room temperature and took approximately 20 min.

Localization and distribution of free-Ca^{2+} in oocyte cytoplasm: The eggs and the fertilized eggs were loaded with fluo-3 acetoxymethyl ester (fluo-3/AM) (Bioturm, USA), the calcium-sensitive and highly specific fluorescent dye by culture in well-equilibrated drops of 5 μM L^{-1} Fluo-3/AM in working medium in CO_2 incubator for 40 min. The Fluo-3/AM-loaded eggs and embryos were rinsed free of outer fluo-3/AM of eggs and embryos in working medium for 5-8 times. Eventually, the localization and distribution of Ca^{2+} in the eggs and the embryos at the defined various developmental stages were carried out using a Confocal Laser Microscope (BIO-RAD, MRC-1024S, USA) with a wavelength of 480 nm.

Experimental design: Experiment 1 was designed to culture bovine eggs in Ca^{2+} culture medium and Ca^{2+}-free culture medium to examine the effect of Ca^{2+} on the embryonic development. Experiment 2 was designed to quantitatively measure the intracytosolic free-Ca^{2+} concentrations of oocytes at MII and embryos at distinct developmental stages (0, 2, 15 and 48 h in Ca^{2+} medium) to investigate the correlation between Ca^{2+} concentrations and oocytes/embryos development. Experiment 3 was designed to determine the spatiotemporal distributions of free-Ca^{2+} within the bovine oocytess (MII) and embryos at different developmental stages (0, 2, 15 and 48 h).

Statistical analysis: In the experiment, there were two treatments for effects of culture medium with or without Ca^{2+} on pronucleus formation and egg cleavage. Analyses were performed on percentage data expressed as number of pronuclear stage embryos or cleaved embryos to IVF oocytes per treatment. There were five treatments for measurements of Ca^{2+} concentrations. The data were submitted to analysis of variance and the differences among the treatments were considered statistically significant at the 5% level.

RESULTS AND DISCUSSION

Experiment 1 effects of Ca^{2+} on *in vitro* development of bovine embryos: The effects of Ca^{2+} on the *in vitro* developmental potential of fertilized eggs were shown in Table 1 and 2. Culturing in Ca^{2+}-free culture medium, no fertilized eggs developed to pronucleus stage and neither cleaved.

The development percentage of embryos in Ca^{2+} culture medium was significantly ($p<0.01$) higher than that in Ca^{2+}-free culture medium. Comparatively, the percentages of pronuclei and cleaved embryos when cultured in Ca^{2+} culture medium were 67.8% (Table 1) and 61.5% (Table 2), respectively.

Table 1: Effects of Ca^{2+} culture medium on pronucleus formation of bovine fertilized eggs

Culture medium	IVF oocytes	Pronucleus embryos (%)
Ca^{2+}	170	117 (67.8)^a
Ca^{2+}-free	183	0 (0.0)^b

Table 2: Effects of Ca^{2+} culture medium on cleavage of bovine fertilized eggs

Culture medium	IVF oocytes	Cleaved embryos (%)
Ca^{2+}	218	142 (61.5)^a
Ca^{2+}-free	229	0 (0.0)^b

Data with different superscript letters (a, b) in a column differ significantly ($p<0.01$)

Experiment 2 Ca^{2+} concentrations of oocytes and embryos at different developmental stages: The measurements of intracytoplasmic free-Ca^{2+} concentrations of the oocytes at MII stage and the embryos at 0, 2, 15 and 48 h of culture in Ca^{2+} culture medium were shown in Table 3. At MII stage, Ca^{2+} concentrations of the oocytes were significantly ($p<0.01$) lower than that at stage 0, 2, 15 and 48 h in Ca^{2+} culture medium, respectively. However, the Ca^{2+} concentrations of fertilized eggs increased at 0 h and peaked at 2 h, significantly ($p<0.01$) higher than the former stage and then considerably ($p<0.01$) decreased at 15 h (pronucleus stage). At 48 h (2-cell stage), the Ca^{2+} concentrations remained the same level ($p>0.05$) as that at 15 h. The trend in the data shown that intracytoplasmic free-Ca^{2+} concentrations of the bovine eggs were lower before fertilization, increase after fertilization and then decrease at pronucleus formation.

Experimental 3 localization and distribution of free-Ca^{2+} within bovine eggs and embryos at various developmental stages: Localization and distribution of free-Ca^{2+} within bovine eggs at various developmental stages were shown in Fig. 1-5. At MII stage, a low density of free-Ca^{2+} were homogenously distributed in the cytoplasm of oocytes (Fig. 1). At the initial time of fertilization, the Ca^{2+} were concentrated at the cortex zone under the egg plasma membrane (Fig. 2). About 2 h later, the Ca^{2+} intensity increased and spread towards the center of egg (Fig. 3). At pronucleus and first cleavage stage, the Ca^{2+} were distributed in whole egg cytoplasm with a weaker intensity (Fig. 4 and 5). Moreover, an intense fluorescence expression shown that the Ca^{2+} also existed in polar bodies.

The activation of MII-staged oocytes, the fertilization, the pronucleus formation and the egg cleavage in mammalian species are arbitrary divisions along a continuum that can be recognized for describing the very early developmental events.

The present study indicated that MII staged bovine oocytes cultured in Ca^{2+} media maintained a low level

Table 3: Concentrations of intracytoplasmic free-Ca^{2+} at different developmental stage in bovine oocytes and embryos

Developmental stage	Oocytes measured	Value (nmol L^{-1})	X±SD (nmol L^{-1})
MII	80	84.99[a]	84.990±8.680
IVC 0 h	80	84.99[a]	120.05±15.43
IVC 2 h	80	120.05[c]	229.09±31.57
IVC 15 h	80	229.09[d]	108.73±13.90
IVC 48 h	80	111.17[c]	111.17±14.18

Data with different superscript letters (a-d) in a column differ significantly ($p<0.01$)

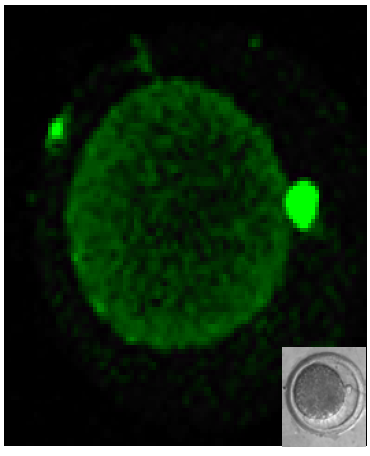

Fig. 1: Intracellular free-Ca^{2+} were homogenously distributed in the cytoplasm of (*in vitro* matured bovine oocyte at MII stage) (x300)

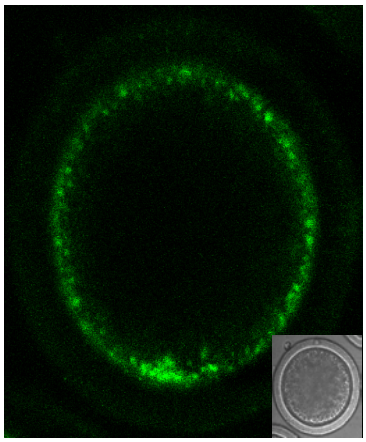

Fig. 2: Intracellular free-Ca^{2+} were concentrated at the cortex zone under the egg plasma membrane of bovine fertilized egg cultured for 0 h (x300)

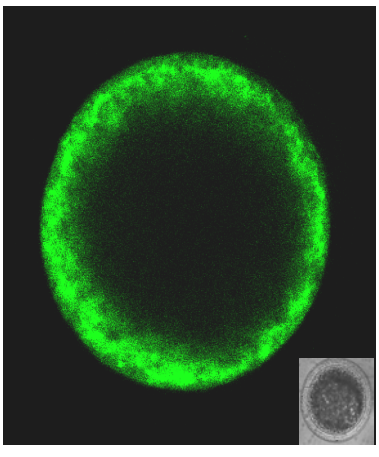

Fig. 3: Intracellular free-Ca^{2+} density increased and spread towards the center of bovine fertilized egg cultured for 2 h (x300)

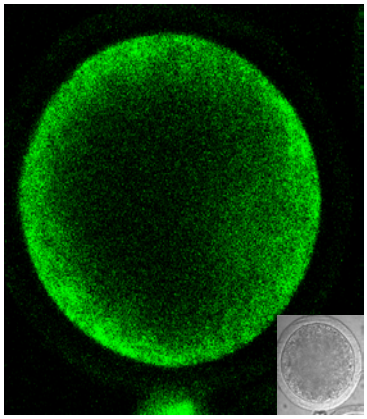

Fig. 4: Intracellular free-Ca^{2+} were distributed in whole egg cytoplasm of bovine zygote at pronucleus stage (x300)

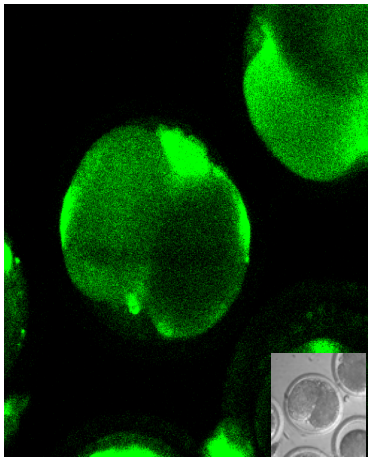

Fig. 5: Intracellular free-Ca^{2+} were distributed in whole cytoplasm of 2-cell embryo and the Ca^{2+} also existed in polar bodies (x200)

(84.99 nM L^{-1}) in cytosolic free-Ca^{2+} concentrations which was consistent with the earlier studies in mouse (Akihiro *et al*., 1999) and porcine. Similar results have been reported that the unfertilized mammalian oocytes arrested at MII maintained a relatively low level (40-100 nM L^{-1}) in cytosolic free-Ca^{2+} concentrations (Jaffe, 1983). These results demonstrated that the low Ca^{2+} level was closely related to the arrest at MII stage in mammalian oocyte. Such a low Ca^{2+} level was probably important for a full maturation of oocyte cytoplasm. It has been known that the potential of eggs to develop ahead after fertilization required a fully-matured cytoplasm (Cheung *et al*., 2000).

The present results indicated that no fertilized eggs formed pronucleus and cleaved when cultured in Ca^{2+}-free media compared with that in the Ca^{2+} media. These implied that the extracellular Ca^{2+} may have an important effect on the initiation of bovine early embryo development. For some species, external Ca^{2+} was demonstrated to contribute to the maintenance of the repetitive Ca^{2+} waves (Deguchi *et al*., 2000; McGuinness *et al*., 1996). In protostomes, sperm-induced intracellular Ca^{2+} increases were immediately abolished by removal of external Ca^{2+} after fertilization (Deguchi *et al*., 1996). These findings suggested that the intracellular Ca^{2+} rise in oocytes might partially be caused by inflow of extracellular Ca^{2+} and so that the extracellular Ca^{2+} was necessary for the very early development of fertilized eggs after fertilization.

As indicated in this study, Ca^{2+} concentrations considerably ($p<0.01$) increased in the eggs at the time of fertilization and uprose to a highest level at 2 h afterwards when cultured in Ca^{2+} media. Subsequently, the Ca^{2+} concentrations of zygotes apparently lowered at pronucleus stage but were still significantly ($p<0.01$) higher than that at MII stage. This result demonstrated that fertilization induced the increase of intracellular Ca^{2+} and that such an increase was essential for oocytes to release from MII stage arrest. With respect to the duration of high Ca^{2+} level, it seemed to be species-specific differences in animals. The research found that the Ca^{2+} concentrations in bovine eggs declined at pronucleus formation stage in the Ca^{2+} media which agreed to that obtained from other mammalian species where the sperm induced high Ca^{2+} concentrations decreased to the level at MII stage and yet the Ca^{2+} oscillations ceased at the time of pronucleus formation (Dumollard *et al*., 2002; Jones *et al*., 1995a, b). However, the Ca^{2+} concentrations as indicated in the present experiment remained higher than that at MII stage after the pronucleus formation till at least the first cleavage completion stage. This comparatively long-lasting high Ca^{2+} concentration might ensure the bovine eggs to be fully activated which benefited the completion of fertilization and the early development of fertilized eggs. The present results may suggest that the steady-state Ca^{2+} concentrations at 2 cell stage are necessary for bovine early embryos to develop ahead. It has been reported that Ca^{2+} transient rise at the activation of oocytes influenced embryonic development and preimplantation (Ozil and Swann, 1995; Vitullo and Ozil, 1992). Therefore, further research is needed for examining the Ca^{2+} concentrations of bovine embryos at subsequent development stages and determining the mechanism of Ca^{2+} increase in triggering the activation of eggs, initiation of development in the fertilization and giving an insight into the mechanism of controlling Ca^{2+} signaling.

The images of fluorescence-labeling showed that the free-Ca^{2+} were dynamically located in different places of cytoplasm at various development phases. At MII stage, a low density of free-Ca^{2+} were distributed in the cytoplasm of ooctyes. After fertilization, the Ca^{2+} signals increased and concentrated at the cortex zone under the egg plasma membrane and then gradually spread towards the center of egg. This evidence may propose that the initial increase of Ca^{2+} concentrations at the cortex area of eggs promote the release of cortical granules. Earlier researches demonstrated that a rise of intracellular free-Ca^{2+} concentrations could mediate a release of cortical granules of eggs (Kline and Kline, 1992). On the formation of pronucleus and the first cleavage stage, the Ca^{2+} were still distributed in egg cytoplasm. In addition, a high intensity of Ca^{2+} were distributed inside the polar bodies (Fig. 1 and 5). This implied that free-Ca^{2+} also existed in nucleoplasm. The roles of Ca^{2+} in nucleoplasm need to be investigated in future research.

CONCLUSION

This research supports the view that there was an increase in intracytoplasmic free-Ca^{2+} concentrations during *in vitro* fertilization in bovine oocytes. Such an increase may be dependent upon an external source of Ca^{2+}. Ca^{2+} is important for bovine oocytes to be activated at fertilization *in vitro*, the formation of pronucleus and the cleavage of early embryos. The free-Ca^{2+} were dynamically located in different sites of cytoplasm at various development phases.

ACKNOWLEDGEMENTS

This research was granted by Natural Science Foundation of Inner Mongolia in China. Researchers were grateful to Experimental Animal Research Center of Inner Mongolia University for supplying the experimental conditions.

REFERENCES

Akihiro, S., O. Shoji, K. Yoshinori and M. Shunichi, 1999. Fertilization, embryonic development and offspring from mouse eggs injected with round spermatids combined with Ca^{2+} oscillation-inducing sperm factor. Mol. Hum. Reprod., 5: 132-138.

Ben-Yosef, D., Y. Oron and R. Shalgi, 1993. Prolonged, repetitive calcium transient in rat oocytes fertilized *in vitro* and *in vivo*. FEBS Lett., 331: 239-242.

Cheung, A., K. Swann and J. Carroll, 2000. The ability to generate normal Ca^{2+} transients in response to spermatozoa develops during the final stages of growth and maturation. Hum. Reprod., 15: 1389-1395.

Cuthbertson, K.R.S. and P.H. Cobbold, 1985. Phorbol ester and sperm activate mouse oocytes by inducing sustained oscillations in cell Ca^{2+}. Nature, 316: 541-542.

Deguchi, R. and K. Osanai, 1994. Meiosis reinitiation from the first prophase is dependent on the levels of intracellular Ca^{2+} and pH in oocytes of the bivalves mactra chinensis and limaria hakodatensis. Dev. Biol., 166: 587-599.

Deguchi, R., H. Shirakawa, S. Oda, T. Mohri and S. Miyazaki, 2000. Spatiotemporal analysis of Ca^{2+} waves in relation to the sperm entry site and animal-vegetal axis during Ca^{2+} oscillations in fertilizing mouse eggs. Dev. Biol., 218: 299-313.

Deguchi, R., K. Osanai and M. Morisawa, 1996. Extracellular Ca^{2+} entry and Ca^{2+} release from inositol 1,4,5-trisphosphate-sensitive stores function at fertilization in oocytes of the marine bivalve mytilus edulis. Development, 122: 3651-3660.

Deng, M.Q., Y.M. Liu and B.Q. Fan, 1995. Parthenogenetic activation of mouse oocytes and intracellular free calcium changes during activation. J. Nanjing Univ., 31: 265-271.

Digonnet, C., D. Aldon, N. Leduc, C. Dumas and M. Rougier, 1997. First evidence of a calcium transient in flowering plants at fertilization. Development, 124: 2867-2874.

Dildy, J.E. and S.W. Leslie, 1989. Ethanol inhibits NMDA-induced increases in free intracellular Ca^{2+} in dissociated brain cells. J. Brain Res., 499: 383-387.

Ducibella, T., D. Huneau, E. Angelichio, Z. Xu and R.M. Schultz *et al*., 2002. Egg-to-embryo transition is driven by differential responses to Ca^{2+} oscillation number. Dev. Biol., 250: 280-291.

Dumollard, R., J. Carroll, G. Dupont and C. Sardet, 2002. Calcium wave pacemakers in eggs. J. Cell Sci., 115: 3557-3564.

Fissore, R.A., J.R. Dobrinsky, J.J. Balise, R.T. Duby and J.M. Robl, 1992. Patterns of intracellular Ca^{2+} concentrations in fertilized bovine eggs. Biol. Reprod., 47: 960-969.

Jaffe, L.F., 1983. Sources of calcium in egg activation: A review and hypothesis. Dev. Biol., 99: 265-276.

Jones, K.T., 2005. Mammalian egg activation: From Ca^{2+} spiking to cell cycle progression. Reproduction, 130: 813-828.

Jones, K.T., J. Caroll, J.A. Merriman, D.G. Whittingham and T. Kono, 1995a. Repetitive sperm-induced Ca^{2+} transients in mouse oocytes are cell cycle dependant. Development, 121: 3259-3266.

Jones, K.T., J. Carroll and D.G. Whittingham, 1995b. Ionomycin, thapsigargin, ryanodine and sperm induced Ca^{2+} release increase during meiotic maturation of mouse oocytes. J. Biol. Chem., 270: 6671-6677.

Kline, D. and J.T. Kline, 1992. Repetitive calcium transients and the role of calcium in exocytosis and cell cycle activation in the mouse eggs. Dev. Biol., 149: 80-89.

Malcuit, C., M. Kurokawa and R.A. Fissore, 2006. Calcium oscillations and mammalian egg activation. J. Cell. Physiol., 206: 565-573.

McGuinness, O.M., R.B. Moreton, M.H. Johnson and M.J. Berridge, 1996. A direct measurement of increased divalent cation influx in fertilized mouse oocytes. Development, 122: 2199-2206.

Mehlmann, L.M., K. Mikoshiba and D. Kline, 1996. Redistribution and increase in inositol 1, 4, 5-trisphosphate receptors after meiotic maturation of the mouse oocyte. Dev. Biol., 180: 489-498.

Mehlmann, L.M., M. Terasaki, L. Jaffe and D. Kline, 1995. Reorganization of the endoplasmic reticulum during meiotic maturation of the mouse oocyte. Dev. Biol., 170: 607-615.

Miyazaki, S., N. Hashimoto, Y. Yoshimoto, T. Kishimoto, Y. Igusa and Y. Hiramoto, 1986. Temporal and spatial dynamics of the periodic increase in intracellular free calcium at fertilization of golden hamster eggs. Dev. Biol., 118: 259-267.

Ozil, J.P. and K. Swann, 1995. Stimulation of repetitive calcium transients in mouse eggs. J. Phys., 483: 331-346.

Ridgway, E.B., J.C. Gilkey and L.F. Jaffe, 1977. Free calcium increases explosively in activating medaka eggs. Proc. Natl. Acad. Sci., 74: 623-627.

Sheu, S. and V.K. Sharma, 1999. A novel technique for quantitative measurement of free Ca^{2+} concentration in rat heart. J. Phys., 518: 577-584.

Shiraishi, K., A. Okada, H. Shirkawa, S. Nakanishi, K. Mikoshiba and S. Miyazaki, 1995. Developmental changes in the distribution of the endoplasmic reticulum and inositol 1,4,5-trisphosphate receptors and spatial pattern of Ca^{2+} release during maturation of hamster oocytes. Dev. Biol., 170: 594-606.

Sun, F.Z., J. Hoyland, X. Huang, W. Mason and R.M. Moora, 1992. A comparison of intracellular changes in porcine eggs after fertilization and electroactivation. Development, 115: 947-956.

Taylor, C.T., Y.M. Lawrence, C.R. Kingsland, M.M. Biljan and K.S. Cuthbertson, 1993. Oscillations in intracellular free calcium induced by spermatozoa in human oocytes at fertilization. Hum. Reprod., 8: 2174-2179.

Vitullo, A.D. and J.P. Ozil, 1992. Repetitive calcium stimuli drive meiotic resumption and pronuclear development during mouse oocyte activation. Dev. Biol., 151: 128-136.