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The role of L-type calcium channels in mouse oocyte maturation, activation and early embryonic development

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ABSTRACT

Calcium ion fluctuation is closely related to the transformation of cell cycle. However, little is known about the function of L-type calcium channel in mammalian oocyte and embryo development. We thus studied the roles of L-type calcium channel in mouse oocyte meiotic maturation, parthenogenetic activation and early embryonic development. We used the antagonist Amlodipine to block L-type calcium channel. Oocytes or zygotes were cultured to different time points with 0 μM, 10 μM, 30 μM and 50 μM Amlodipine. Then we checked the rate of first polar body extrusion, spindle formation, asymmetric division parthenogenetic activation and early embryo cleavage. The results showed that Amlodipine treatment did not affect germinal vesicle breakdown, but caused disruption of cytoskeleton organization, symmetric division, formation of mature oocytes with a large polar body, or reduced the first polar body extrusion, depending on its concentrations. Amlodipine treatment also resulted in decreased parthenogenetic activation and arrested early embryonic development. Overall, these data suggest that proper function of L-type calcium channel is critical for oocyte maturation, activation, and early embryonic development.

1. Introduction

Proper cell cycle progression and accurate segregation of chromosomes ensures that each daughter cell receives exactly one copy of the genome during each cell cycle. In meiosis, two successive divisions occur with only one round of DNA replication, thereby producing haploid gametes on which the genetic stability of species depends. Any error in this process will result in failure of fertilization or developmental disturbances that are manifested in genetic disorders [1–3]. It is well known that oocyte provides the components (such as mRNA and proteins) to support embryo's development until the embryos activate their own genome [4,5]. The quality of mature oocyte directly determines the fate of early embryo [6–8]. Although at present, oocyte *in vitro* maturation (IVM) system has been improved, the quality of IVM oocytes is not the same good as those matured *in vivo* [9–11]. In the process of oocyte maturation, the nuclear envelope breaks down (germinal vesicle breakdown, GVBD) and the oocyte undergoes accurate segregation of homologous chromosomes during meiosis I, followed by the extrusion of the first polar body (PB1), which is called nuclear maturation [12,13]. Nonetheless, the maturing oocyte also needs to undergo cytoplasmic maturation, which may not be consistent to nuclear maturation [14,15]. During meiotic maturation, some intracellular components such as the spindle, mitochondria, and cortical granules (CGs) change in position, which is significant for subsequent embryo development [16–18].

Calcium ions generally show dynamic changes in many animal cells, and its concentration fluctuations (Ca^{2+}oscillations) are closely related to transformation of the cell cycle [19,20]. Oocyte maturation and activation are also regulated by calcium ion

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concentration fluctuations [21]. Increasing intracellular calcium ion concentration was observed at fertilization and parthenogenetic activation of oocytes [22–26]. Furthermore, intracellular calcium and external calcium also regulate oocyte meiotic maturation. L-type calcium channel is a kind of double hydrogen pyridine sensitivity (DHP) voltage-dependent calcium channel, and widely exists in various kinds of excitable cells and non-excitable cells [27]. In addition, it mediates cellular functions, such as excitation contraction coupling, signal transduction, the neurotransmitter release. Moreover, it has vital roles in cardiac muscle, smooth muscle, skeletal muscle, brain and endocrine tissue [28]. Oocyte meiotic resumption marked by GVBD and oocyte activation are finely regulated in different species [29]. But little is known about the function of L-type calcium channel in mammalian oocyte maturation and early embryonic development.

In this research, we investigated the roles of L-type calcium channel in mouse oocyte meiotic maturation, parthenogenetic activation and early embryonic development by using its antagonist. Amlodipine is a calcium channel blocker which is widely used in the treatment of hypertension and angina [30]. We found that oocytes in Amlodipine-treated groups showed reduced first polar body extrusion, symmetric division or large PB1 extrusion, reduced parthenogenetic activation, and retarded early embryonic development, indicating important functions of L-type calcium channel in these processes.

2. Materials and methods

2.1. Antibodies and chemicals

All antibodies and chemicals used in the experiments were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA) unless indicated otherwise. Amlodipine was prepared in DMSO at a stock concentration of 10 mM.

2.2. Oocyte collection and culture

Animal care and use were conducted in accordance with the Animal Research Committee guidelines of Qingdao Agricultural University. Immature oocytes with clear germinal vesicle (GV) were collected from ovaries of 6-week-old female ICR white mice in M2 medium, supplemented with 2.5 μM Milrinone which prevented GVBD. After washing, oocytes were cultured in M16 medium under liquid paraffin oil at 37 °C in an atmosphere of 5% CO2 in air. At different time points after culture, oocytes were collected for further experiments. The oocyte maturation dynamics including GV(0 h), GVBD (2 h), MI (8 h), AI-TI (9.5 h), and MII (13 h) was referenced from a previous publication [31].

2.3. Amlodipine treatment experiments

Fully-grown oocytes with GV in the middle were selected and divided into four similar quality groups. Then oocytes were cultured to different time points with 0 μM, 10 μM, 30 μM and 50 μM Amlodipine, respectively. Bright field images were captured by a DM IRB inverted microscope (Leica Microsystems, Bannockburn, IL). Polar body diameter was determined with ImageJ (NIH) by taking a line measurement at the widest position.

2.4. Immunofluorescence and confocal microscopy

Immunofluorescence was performed as described previously [31,32]. Briefly, oocytes were fixed in freshly prepared 4% paraformaldehyde for 30 min, and then permeabilized with 1% Triton X-100 in phosphate-buffered saline (PBS) for 20 min, followed by blocking in 1% bovine serum albumin (BSA) for 1 h at room temperature. Then they were incubated with Mouse monoclonal anti-α-tubulin FITC antibody (1:500, SantaCruz) for 4 h. Or oocytes were incubated with Phalloidin-TRITC (1:500) for 1 h at room temperature. After washed in washing buffer (PBS containing1/1000 Tween20 and 1/10000 TritonX-100), oocytes were stained with Hoechst 33342 (0.5 μg/ml) prepared in PBS for 15min.

The staining of CGs was performed as described previously [33]. Briefly, oocytes were exposed to 0.25% pronase for 1–2min to remove the zonapellucida. Zona-free oocytes were washed three times in M2 medium and fixed with 3.7% paraformaldehyde in M2 (pH 7.4) for 40 min at room temperature. Then they were washed three times in blocking solution (M2 medium with 0.3% BSA and 100 mM glycine), permeabilized in M2 medium with 0.1% Triton X-100 for 5min, blocked overnight at 4 °C or 1 h at room temperature. After washing twice, CGs were stained for 1 h in 100 μg/mL of FITC-labeled lens culinaris agglutinin (FITC-LCA, FL-1041, Vector Laboratories).

These stained oocytes were mounted with anti-fade medium (Vector Laboratories, Burlingame, CA, USA) in glass slides, and then observed with a laser scanning confocal microscope (Leica TCS SP5 II, Wetzlar, Germany).

2.5. Superovulation and parthenogenetic activation

Female mice were administrated intraperitoneal injections of 10 IU pregnant mare's serum gonadotropin (PMSG) followed 44–48 h later by human chorionic gonadotropin with 10IU (hCG) (both from Ningbo Hormone Product Co. Ltd., Cixi, China) [34]. After 14 h of hCG administration, MII oocytes were collected, and then treated in CZB with different concentrations of Amlodipine as described above after removal of cumulus cells, and activated by 10 mM $SrCl_2$ (10025-70-4, Sangon Biotech, Shanghai) dissolved in Ca^{2+} free CZB medium. After 5–6 h, the activation rate of parthenogenetic embryos was observed.

2.6. Zygote collection and embryo culture

Superovulation was performed as described above. After hCG administration, the female mice were mated with adult ICR males overnight to obtain early embryos. Zygotes were collected from oviducts at next day after post-hCG injection 20 h, and cultured in CZB medium supplemented with different concentrations of Amlodipine for 96 h. Embryo morphology was observed and images were acquired using a Nikon SMZ150.

2.7. RNA extraction and quantitative real time PCR (qRT-PCR)

About 150 oocytes were collected from mice and total RNA was extracted by using PureLink RNA Mini Kit (Invitrogen). Reserve transcription was conducted with *TransScript* One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen). All the primers were listed in supplementary file (supplementary table 1). The SYBR Premix Ex Taq kit (Takara) was used in Applied Biosystems 7500 Sequence Detection System. Amplification was performed in 20 μL reactions containing 10 μL of SYBR Green Master Mix, 7.2 μL of RNase free H_2O, 0.4 μL of primers (10 μM), 0.4 μL of ROX Reference Dye 2 and 2 μL of cDNA. PCR amplification conditions were as follows: reaction was initialed at 95 °C for 10 min, followed by 40 cycles of denaturing at 95 °C for 15 s, annealing at 60 °C for 30 s, and extension at 72 °C for 20 s. The threshold cycle values were calculated with mean ± SD.

Table 1
Expression of voltage-dependent Ca^{2+} channels in mouse oocytes.

Gene	GV oocyte Ct[a]	MI oocyte Ct	MII oocyte Ct
Cacnalc	33.89 ± 0.52	35.83 ± 0.03	35.67 ± 0.23
Cacnald	28.76 ± 0.12	30.57 ± 0.32	30.81 ± 0.07
Cacnals	ND[b]	ND	ND
Cacnalf	ND	ND	ND
Cacnb4	28.94 ± 0.03	31.42 ± 0.53	30.06 ± 0.06
Cacnb2	29.11 ± 1.24	29.72 ± 0.22	30.45 ± 1.05

[a] Ct, threshold cycle.
[b] ND, not detected.

2.8. Statistical analysis

All experiments were conducted at least three times. Results were analyzed by GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA, USA). The significance of differences between groups was analyzed by the Chi-square test and p-values less than 0.05 were considered statistically significant.

3. Results

3.1. L-type calcium channel related genes are expressed in mouse oocytes

To confirm whether the L-type channel was present in mouse oocytes, we searched the database for different L-type calcium channels subunits and six kinds of subunits encoded by gene *Cacna1c, Cacna1d, Cacna1f, Cacna1s, Cacnb4* and *Cacnb2* were found. Total RNA of different stage oocytes were extracted and the mRNA expression levels were quantified by qRT-PCR. According to the Ct values, the expression levels of *Cacna1c*, *Cacna1d, Cacnb4* and *Cacnb2* were identified (Table 1). While the expression levels of *Cacna1s* and *Cacna1f*, by contrast, were not detected. While primers for *Cacna1s* and *Cacna1f* were identified positive in cDNA amplifications of TM4 cells and mouse embryonic fibroblast (MEF) cells. These data indicated that L-type calcium channel was indeed present in mouse oocytes.

3.2. Amlodipine reduces the first polar body extrusion

The effect of Amlodipine on oocyte meiotic maturation was shown in Fig. 1A. In order to get as many synchronized oocytes as possible, 2.5 μM Milrinone was added to the medium to prevent GVBD while collecting oocytes [31,35]. We only collected the cumulus-free fully-grown oocytes for maturation in medium containing 0 μM, 10 μM, 30 μM and 50 μM Amlodipine. As shown in Fig. 1B, Amlodipine treatment did not affect GVBD. After 13 h of maturation culture, we examined the rate of the first polar body extrusion (PBE). As shown in Fig. 1C, PBE in 10 μM Amlodipine group did not have significant difference compared with the control. With the increasing concentration of Amlodipine, the rate of PBE significantly decreased compared to the control, indicating that L-type calcium channel plays critical roles in mouse oocyte maturation *in vitro*.

3.3. Amlodipine treatment disrupts mouse oocyte asymmetric division

Of all oocytes with the first polar body emission, 100% underwent an asymmetric division in the control group, producing a large egg with a small polar body (arrow indicates typical image in Fig. 1A). However, oocytes treated with Amlodipine extruded much bigger polar bodies (arrowheads indicate typical image in Fig. 1A). In 30 μM or 50 μM Amlodipine-treated groups, nearly 40% and 50% mature oocytes divided symmetrically and produced big polar bodies, respectively (Fig. 2A). Diameter measurement of cells revealed significantly increased polar body size in Amlodipine-treated groups (Fig. 2B). To present clear cytoskeleton dynamic changes, oocytes were stained with anti-α-tubulin FITC and Hoechst 33342, and it was showed that Amlodipine treatment did undergo symmetric division, and in each similar-sized daughter cells, a typical spindle was formed (Fig. 2C). All these results showed depletion of calcium by blocking L-type calcium channel disrupts mouse oocyte asymmetric division.

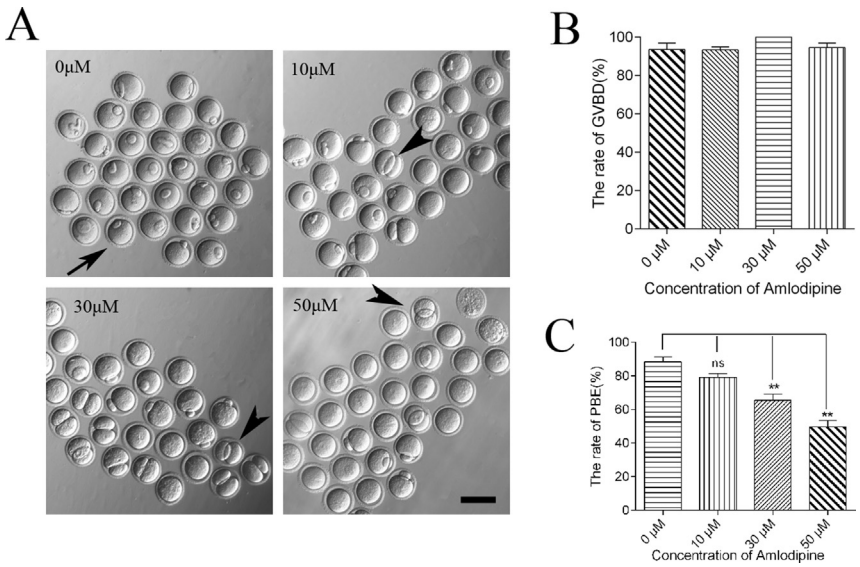

Fig. 1. The L-type calcium channel antagonist Amlodipine reduces the first polar body extrusion. A. Representative images of control and Amlodipine-treated oocyte maturation *in vitro*. Arrow indicates a typical oocyte with the normal size of the first polar body. Arrowhead indicates a typical oocyte with a big polar body. B. Rates of germinal vesicle breakdown (GVBD) after treatment with different concentrations of Amlodipine. There was no significant difference among them. C. Rates of the first polar body extrusion (PBE) after treatment with different concentrations of Amlodipine. With the increase of Amlodipine concentration, the rate of PBE decreased. ** indicates significant difference ($P < 0.01$). Scale bars = 100 μm, ns = no significant difference.

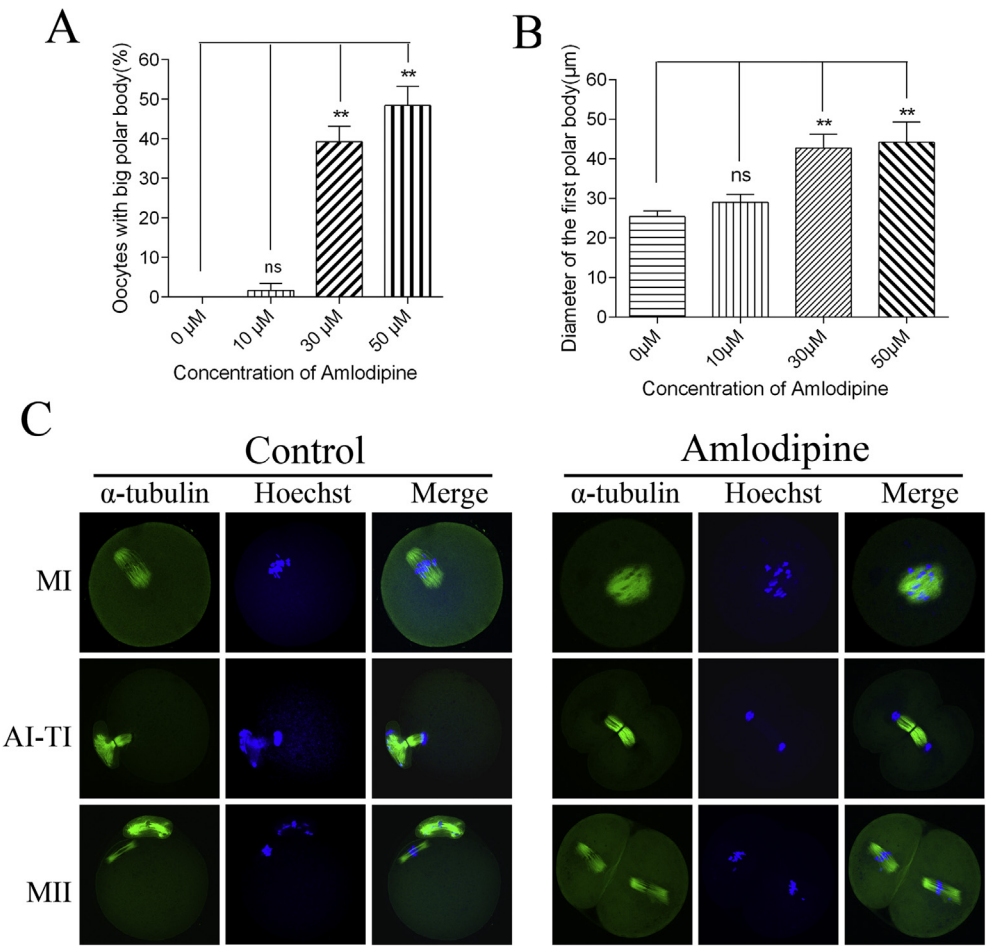

Fig. 2. The L-type calcium channel antagonist Amlodipine disrupts asymmetric division of mouse oocyte. A. Amlodipine-treated oocytes showed a higher rate of big first polar body compared with control oocytes. B. Measurement of the first polar body diameter at its widest position. The size of first polar body in Amlodipine-treated oocytes was significantly larger than that in control oocytes. C. Confocal micrographs of control oocytes and Amlodipine-treated oocytes displaying the first polar body and spindles. Control oocytes and Amlodipine-treated oocytes proceeded through metaphase I (MI), anaphase/telophase I (AI/TI), and established a meiotic arrest at metaphase II (MII). Typical Amlodipine-treated oocyte showed a big first polar body. All oocytes were stained with anti-tubulin antibody (green) and Hoechst 33342 (Blue). Scale bars = 100 μm, ** indicates significant difference ($P < 0.01$), ns = no significant difference. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. Amlodipine affects normal spindle formation and migration, actin cap formation and cortical granule distribution

Since Amlodipine-treated oocytes extruded big polar bodies, we carefully analyzed cytoskeleton changes. First we employed anti-α-tubulin FITC antibody immunofluorescent staining to demonstrate the organization and migration of the spindle, as well as cell polarization formation. As shown in Fig. 3A, in MI stage, normal spindle was formed in the control group while abnormal spindle was observed in the Amlodipine-treated group. The percentage of abnormal spindle in 50 μM Amlodipine group was significantly higher than that in control group (Fig. 3B), indicating that blocking L-type calcium channel disrupts the spindle formation. Around 9 h of culture, spindles in mouse oocytes moved to the cortex at roughly late MI stage (Fig. 3C). However, in high dose of Amlodipine-treated group, spindle was still located in the center of oocyte, even some had a normal shape (Fig. 3C, right). Oocytes treated with 30 μM Amlodipine (62.11 ± 10.13%, $n = 130$ vs control group 32.3 ± 4.29%,$n = 122$; $P < 0.05$) or 50 μM Amlodipine (63.13 ± 7.86%, $n = 122$ vs control group 32.3 ± 4.29%,$n = 122$; $P < 0.05$) displayed centrally-located spindles (Fig. 3D), indicating that blocking L-type calcium channel by Amlodipine deteriorates peripheral spindle migration.

The F-actin dynamics was also investigated. Especially, the actin cap, as a polarization criterion, was also observed to clarify whether oocyte polarity was disrupted [36]. As shown in Fig. 3E, the chromosomes of control group had already moved to the cortex and formed an actin cap at the late MI stage after 9 h of culture. But in the experimental groups, it was hard to find a typical actin cap. Also, we probed the cortical granule (CG) distribution, since cortical granule-free domain (CGFD) was not only a criterion of assessing cytoplasmic maturation, but also a feature of oocyte polarization [37]. After Amlodipine treatment, CGs were still interspersed in the cortical cytoplasm, without CGFD formation (Fig. 3F). Therefore, oocyte polarity was disrupted by Amlodipine treatment, which indicates that the failure of polarity is related to cytoplasmic Ca^{2+} concentration.

3.5. Amlodipine affects parthenogenetic activation

Next we investigated whether Amlodipine-treated *in vivo* matured oocytes could be parthenogenetically activated. With the increasing concentration of Amlodipine, less parthenogenetically activated oocytes and more dead oocytes were observed (Fig. 4A). As shown in Fig. 4B, the rate of parthenogenetic activation, even in 10 μM Amlodipine-treated group, was significantly lower than that

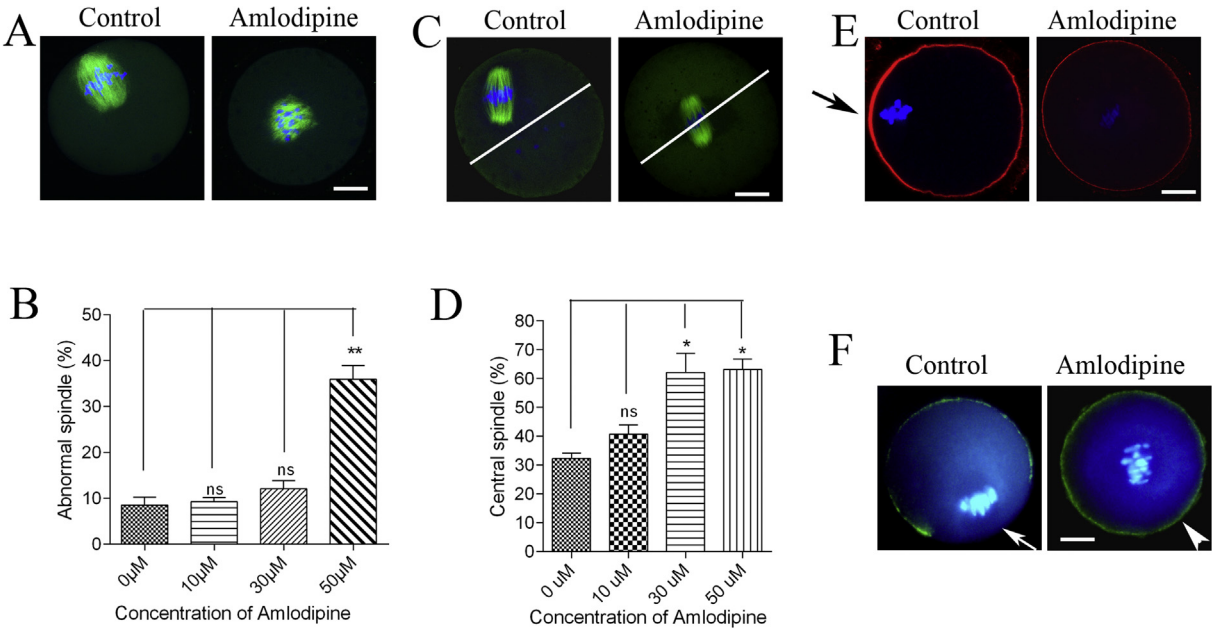

Fig. 3. Amlodipine affects the spindle migration and actin cap formation. A. Typical defective spindle images of Amlodipine-treated oocytes at metaphase I were shown. Oocytes were stained with anti-tubulin antibody (green) and Hoechst 33342 (Blue).B. Rates of oocytes with abnormal spindles in the high concentration Amlodipine-treated group was significantly increased compared to the control group. C. The spindle moved to the cortex after 9 h of culture in the control group, but in the middle in a high concentration Amlodipine-treated group, even the normal shape spindle was still centrally located. Oocytes were stained with anti-tubulin antibody (green) and Hoechst 33342 (Blue).D.Rates of centrally-located spindles in oocytes after 9 h of culture. *indicates significant difference ($P < 0.05$). E.After9h of culture, an actin cap had formed in the control group (arrow), but no clear actin cap was observed in the Amlodipine-treated group. Oocytes were stained with TRITC-Phalloidin (red) and Hoechst 33342 (blue). F. Amlodipine affected cortical granule migration and formation of the cortical granule-free domain (CGFD). At the MII stage, oocytes in the control group formed CGFD normally (arrow), while there is no CGFD formation in the Amlodipine-treated oocytes (arrowhead). Oocytes were stained with Lectin lens culinaris (green) and Hoechst 33342 (blue). All scale bars = 20 μm, ** indicates significant difference ($P < 0.01$), ns = no significant difference. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

in the control. In addition, dead oocytes are found from 30 μM and higher concentration Amlodipine-treated groups. Therefore, inhibition of L-type calcium channel by lower concentration of Amlodipine reduces parthenogenetic activation and high concentration of Amlodipine can cause oocyte death.

3.6. Amlodipine impairs early embryonic development

We further investigated the effects of Amlodipine treatment on embryonic development of zygotes. Zygotes were collected from oviducts at next day after hCG injection, termed as 0 h, and copulation. We cultured them to 24 h, 44 h and 96 h to examine the development of two-cell embryos, four-cell embryos and blastocysts with Amlodipine treatment consistently. Embryos in two-cell stage (black arrow), four-cell stage (black arrowhead) and blastocyst stage (white arrowhead) were shown in Fig. 5A. With 10 μM Amlodipine treatment, most zygotes developed to the two-cell stage, no difference with control group (Fig. 5B). But in 50 μM groups, the rate of two-cell embryos was significantly lower than that in control, and some 2-cell stage embryos died (Fig. 5A). By

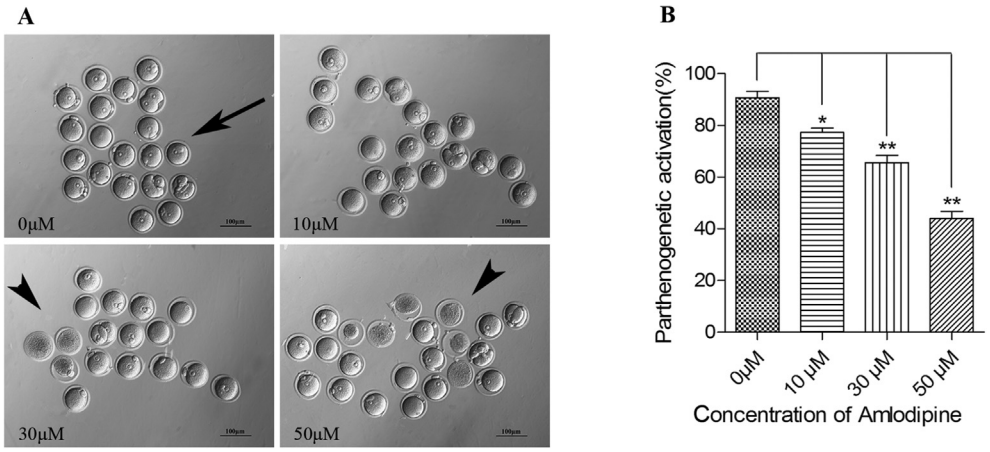

Fig. 4. Amlodipine reduces the rate of parthenogenetic activation. A. Typical images of control and Amlodipine-treated groups were shown after parthenogenetic activation *in vitro*. Arrow indicates an oocyte with a pronucleus and arrowhead indicated a dead oocyte. B. Rates of parthenogenetic activation after treatment with different concentrations of Amlodipine. With the increase of Amlodipine concentration, the rate of activation decreased. ** indicates significant difference ($P < 0.01$), *indicates significant difference ($P < 0.05$), ns = no significant difference. Scale bars = 100 μm.

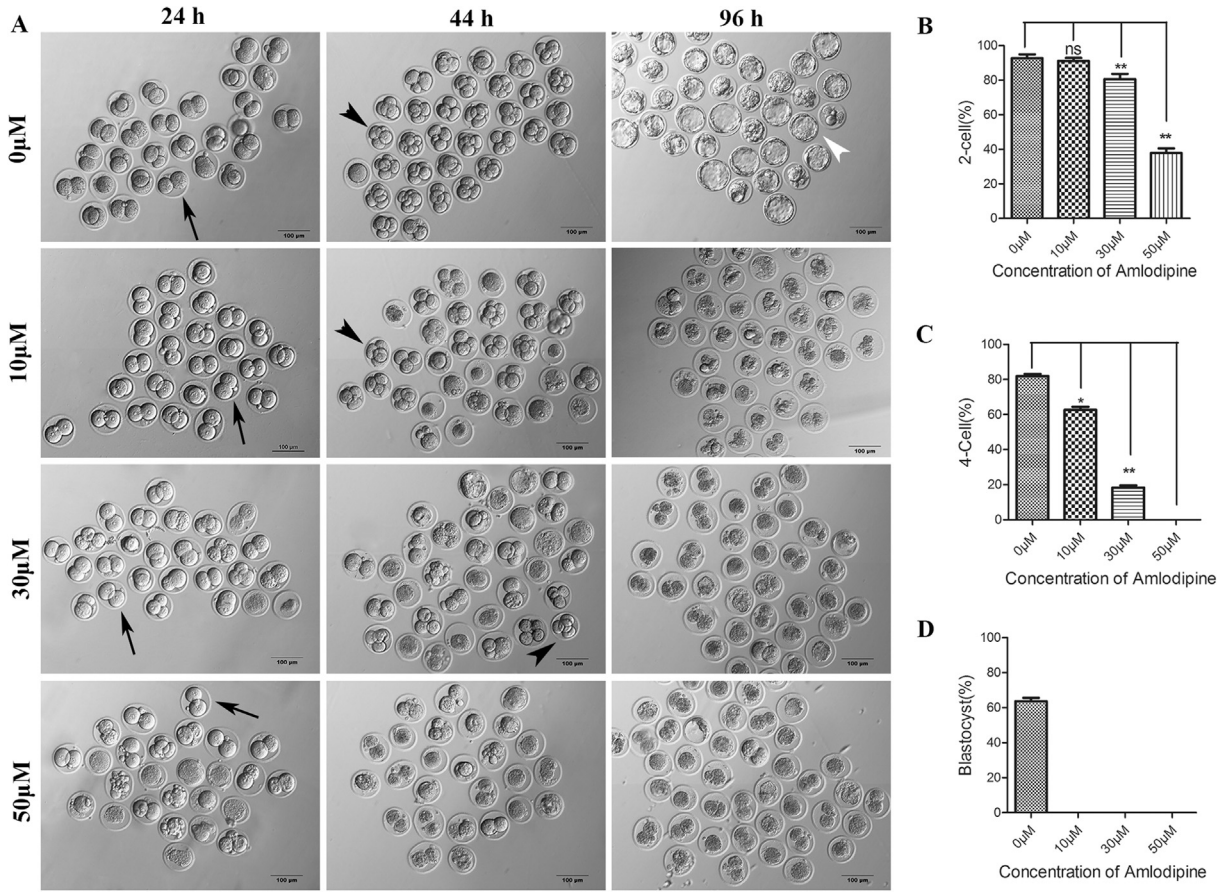

Fig. 5. Amlodipine impairs early embryonic development. A. Morphology of embryos cultured with different concentrations of Amlodipine. Arrow indicated 2-cell embryo, black arrowhead indicated 4-cell embryo and white arrowhead referred to the representative blastocyst. Embryonic morphology was observed and images were acquired at 24 h, 44 h and 96 h of culture. B. The rate of 2-cell embryos after treatment with different concentrations of Amlodipine at 24 h. There was no significant difference among the control and 10 μM Amlodipine-treated groups. However, 50 μM Amlodipine-treated group showed a significant lower rate of 2-cell embryos. C. The rate of 4-cell embryo after treatment with different concentrations of Amlodipine at 44 h. With the increase of Amlodipine concentrations, the rate of 4-cell embryos decreased significantly. D. The rate of blastocysts after treatment with different concentrations of Amlodipine at 96 h. No one exposed to Amlodipine could develope to blastocyst stage. ** indicates significant difference ($P < 0.01$), ns = no significant difference, Scale bars = 100 μm.

44 h, a majority of the control embryos reached four-cell stage. But in the Amlodipine-treated groups (10 μM and 30 μM) only a small fraction developed to the four-cell stage, and the difference was significant as shown in Fig. 5C. By 96 h, even in 10 μM Amlodipine, no one could develop to the blastocyst stage (Fig. 5D). All these data suggest that blocking of L-type calcium channel retards early embryonic development.

4. Discussion

So far, numerous reports about functions of L-type calcium channels focus on mitosis of somatic cells. In this study, we aimed to investigate the possible functions of L-type calcium channels in mouse oocyte meiotic maturation by using its antagonist Amlodipine. Our results showed that Amlodipine affects oocyte spindle, morphology and movement, actin cap formation, and CG migration, resulting in symmetric division, failed polar body extrusion, and impaired parthenogenetic activation, as well as early embryonic development inhibition.

During *in vitro* culture of mouse oocytes, nuclear maturation completes first, while the oocyte cytoplasm may not have sufficient time to complete maturation. The study on human oocytes also confirms this point [38]. It is demonstrated that there is a high level of asynchrony between nuclear maturation and cytoplasmic maturation. In addition, it also proved that incomplete CG migration, polarity loss and large polar bodies are indicators of poor oocyte quality [39]. In our study, antagonist of L-type calcium channels also causes big polar body formation and failure of peripheral CG migration and polarity formation. Hence, L-type calcium channels may be a key regulator of oocyte quality by regulating Ca^{2+}.

Previous studies suggested that the actin capping protein mainly localizes in the oocyte cytoplasm during maturation, then it is essential for actin cytoskeleton machinery, correct asymmetric spindle migration and polar body extrusion [40]. L-type calcium channels may coordinate with capping protein to affect the formation of actin cap. It has been shown that Fmn2-Arp2/3-orchestrated cytoplasmic streaming breaks the symmetrical division, maintains the oocyte polarity, and transports the spindle to the cortex [41,42]. How L-type calcium channel participates in this process needs further research.

Oocyte activation requires Ca^{2+} rise, and subsequent calmodulin and calmodulin-dependent protein kinase pathway. The inhibition of L-type calcium channel causes failure of oocyte activation, suggesting that it is important for Ca^{2+} rise in oocyte. Meiosis initiation also requires calcineurin. It is known that calcineurin subunits, CnA and CnB, localize in the cortex area of MII oocytes, *in vitro* fertilized and parthenogenetically activated oocytes. Besides, calcineurin

participates in the process of pig oocyte activation [43]. Whether L-type calcium channels are associated calcineurin needs further clarification.

Calcium is also important for mitotic progression and cell survival of early embryos, and L-type calcium channels appear to be the key regulator of calcium in mouse embryos, since Amlodipine caused mitotic failure and even embryo death, depending on concentrations. It is demonstrated that the mTORC2/Akt1/Girdin signaling pathway is significant for regulating F-actin in mouse fertilized eggs [44]. Existing research showed that maternal Yap1-knockout embryos block in the two-cell stage and develop into the four-cell stage in a longer time. Amlodipine treatment obtained similar results. It appears that L-type calcium channel functions in early embryos, and it may plays its roles by regulating intracellular calcium, and thus affect cytoskeleton function, cell cycle progression and even cell survival.

In conclusion, our study indicates that L-type calcium channel plays important roles in oocyte meiotic maturation, parthenogenetic activation and early embryonic development.

Author contribution

Gui-Fang He and Lei-Lei Yang contributed equally to this work.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.theriogenology.2017.07.012.

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