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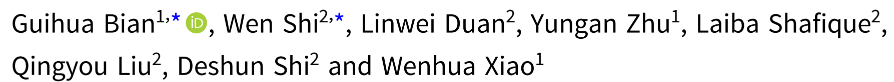
Effect of milrinone on the meiosis resumption and cytoplasm maturation of buffalo oocytes



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Summary

Buffalo has many excellent economic traits and it is one of the greatest potential livestock. Compared with cattle, buffalo has poorer reproductivity, it is of great significance to improve the development potential of oocytes. Buffalo oocyte *in vitro* maturation (IVM) has been widely used in production, but the poor development ability of bovine oocytes IVM limits the development of buffalo reproductivity. Milrinone as a phosphodiesterase inhibitor could affect the maturation of oocytes in goat and mice, but there have been few reported studies in water buffalo. To optimize buffalo oocyte *in vitro* maturation systems, the effects of phosphodiesterase inhibitor (milrinone) on pre-maturation culture of buffalo oocytes were investigated in this study. Buffalo cumulus–oocyte complexes (COCs) were cultured in medium with different concentrations (0, 12, 25, 50 and 100 mol/l) of milrinone for different times (0, 4, 8, 12, 16, 22 and 24 h). The results showed that the buffalo COCs nuclear maturation process could be inhibited by milrinone (25–100 mol/l) in a dose-dependent manner. The inhibitory effect of milrinone on *in vitro* maturation of buffalo oocytes did not decrease with the extension of time. This indicated that milrinone can be used as a nuclear maturation inhibitor during the maturation process in buffalo oocytes. In addition, milrinone can inhibit the effect of follicle stimulating hormone (FSH)-induced IVM of buffalo oocytes, but with time FSH partially eliminated the inhibition. Therefore, inhibition of milrinone on the nuclear maturation of buffalo oocytes was reversible, and buffalo oocytes can mature normally after the inhibition is lessened.

Introduction

Several intrinsic factors for the species, including delayed puberty, higher age at calving, long postpartum anestrus period, long calving interval, lack of overt sign of heat, and low conception rate eventually results in overall low reproductive efficiency in buffalo species (Kumar and Anand, 2012). Furthermore, female buffaloes have few primordial follicles and a high rate of follicular atresia (Rubessa *et al*., 2019). These problems lead to the low competency of buffalo superovulation (SO) and embryo transfer (ET). Therefore, *in vitro* embryo production (IVEP) technology has attracted more and more attention to the rapid reproduction of buffalo superior germplasm (Gasparrini, 2002). Meiosis inhibitors such as cAMP and cGMP exist in the follicular micro-environment and retain the nuclei of oocytes in the germinal vesicle (GV) during the oestrus cycle. Resumption of meiosis in mammalian oocytes is stimulated by increase in gonadotrophins such as luteinizing hormone (LH). During meiosis, the nucleus of the oocyte undergoes processes such as germinal vesicle breakdown (GVBD) or chromosome condensation, then it develops to the middle stage of the first meiosis (MI). Meiosis I is completed after the release of the first polar body (PBI) and, at the middle of meiosis II, oocytes further developed and become stagnates. Only second meiosis (MII) oocytes can complete fertilization and embryonic development (Jia *et al*., 2013).

Numerous studies have indicated that cAMP blocks the resumption of meiosis in mammalian oocytes (Kalinowski *et al*., 2004; Wang *et al*., 2016). By reducing the cAMP level in cytoplasm, this can trigger the resumption of meiosis and promote the occurrence of GVBD (Thomas *et al*., 2004; Wang, *et al*., 2016). Natriuretic peptide and cGMP in follicles prevent the resumption of meiosis by inhibiting the hydrolysis of intracellular cAMP, and gonadotropin increases sharply before ovulation, and can reverse this process. Phosphodiesterase (PDE) is a key enzyme that regulates intracellular cAMP and cGMP levels and plays an important role in the regulation of oocyte maturation and cleavage (Gilchrist *et al*., 2016). PDE has 11 isoenzymes with the ability to hydrolyze cAMP or cGMP or both nucleotides (Gilchrist *et al*., 2016; Shafiee-Nick *et al*., 2017). Maurice and Haslam's study showed that PDE3A is a cAMP hydrolase inhibited by cGMP, one of the most important phosphodiesterase (PDE) in oocytes (Maurice and Haslam, 1990). The decreasing level of cGMP in

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oocyte increased the oocyte specific phosphodiesterase (PDE3A) activity, which should be an important condition for the resumption of oocyte meiosis process. PDE3A activity in the cytoplasm of developing oocytes was significantly lower than that of fully developed oocytes (Gershon *et al.*, 2019). The blocking effect of PDE3 inhibitors on oocyte meiosis maintenance has been demonstrated in many mammalian species, including rhesus monkey (Jensen *et al.*, 2002), bovine (Alam *et al.*, 2018), mouse (Romero and Smitz, 2010), sheep (Gharibi *et al.*, 2013), human (Nogueira *et al.*, 2003). Milrinone is a selective PDE3 inhibitor that reduces PDE3 activity, and then ceases development at the GV stage, finally resulting in the delay of oocyte nuclear maturation (Alam *et al.*, 2018). Lamb oocytes were cultured with milrinone at different concentrations (0, 50, 75, and 100 μM) and at different times intervals (3, 6, and 9 h), and compared with other treatments, oocytes cultured for 6 h with 75 μM milrinone had the highest blastocyst rate (Wang *et al.*, 2016).

To our knowledge, few studies have reported on using the milrinone to regulate the maturation process of *in vitro* buffalo oocytes. This study was designed to find the effect of the PDE subtype-specific inhibitor milrinone on the meiotic maturation of buffalo oocytes, and further effects on the fertilization process of IVM buffalo oocytes.

Materials and methods

Materials

Animal welfare was approved by the Animal Ethics Committee of Guangxi University.

Reagents and media

Tissue culture medium 199 (TCM-199) was produced by Gibco (Grand Island, NY, USA), all other inorganic salts and biochemical reagents were produced by Sigma (Sigma-Aldrich®, St. Louis, MO, USA). Buffalo ovarian preservation solution: physiological saline containing K^+, Ca^{2+} and Mg^{2+}. Oocyte washing liquid (CCM): TCM-199 + 5.0 mmol/l $NaHCO_3$ + 5 mmol/l HEPES + 2% oestrous bovine serum (OCS). Basic buffalo oocyte maturation medium (M) included TCM-199, 26.2 mmol/l $NaHCO_3$ and 5 mmol/l HEPES. Fixed solution of glacial acetic acid (analytical pure) contained anhydrous ethanol (analytical pure 1:3). Resorcin blue was prepared in 40% acetic acid solution, the final concentration was 1%. Dyeing liquor was stored at 4°C, and filtering by centrifugation was necessary before use. Milrinone was dissolved in dimethyl sulphoxide (DMSO) with a storage concentration of 20 mM, and milrinone storage solution was stored at −20°C. Freezing base solution: TCM-199–HEPES buffer containing 15% FCS. Freezing equilibrium solution: 7.5% EG, + 7.5% DMSO + 0.5 mol/l sucrose + freezing base solution. Freeze solution (EDS33): 16.5% EG + 16.5% DMSO + 0.5 mol/l sucrose + freeze base solution. Defrost solution 1 (WM1): freezing base solution + 0.5 mol/l sucrose; defrost solution 2 (WM2): freezing base solution + 0.45 mol/l sucrose; defrost solution 3 (WM3): freezing base solution + 0.25 mol/l sucrose. In the experiment, 60 mg/l penicillin and 100 mg/l streptomycin were added in all the media. All reagents were filtered through a 0.22-μm microporous membrane.

Methods

Oocyte collection and maturation culture

China swamp buffalo ovaries were collected from a Nanning slaughterhouse, then transported to the laboratory in a thermos flask containing saline solution at 37°C within 4 h. Oocytes from 2–6 mm follicles were aspirated using a 10-ml syringe with a 12-gauge needle. The cumulus–oocyte complexes (COCs) with homogeneous cytoplasm and intact granulosa cells were selected under a stereomicroscope and washed twice with CCM. Oocytes were cultured in basic maturation solution (M), and the culture density of oocytes was ~100 cells per 1.5 ml. Then oocytes were *in vitro* matured in 38.5°C and the maximum saturated humidity in the incubator for 24 h.

In vitro *fertilization (IVF) of oocytes*

The frozen semen were thawed in a 37°C water bath, suspended in improved Tyrode's solution for 30 s, and checked for sperm motility by a microscope and qualified (Nikon, Japan). Then the supernatant was removed by centrifugation for 5 min (1500 r/min). The collected sperms (1×10^6/ml, in a 10 ml glass centrifuge tube) were used for IVF with the maturation buffalo oocytes in a 100-μl micropipette with 10–15 oocytes per drop in the oil-covered fertilization solution droplet. Finally, it was placed in a CO_2 incubator for IVF.

In vitro *embryo culture*

After IVF, the buffalo oocytes or zygotes were cultured for 24 h or 48 h in a monolayer of granulosa cells. The culture solution was refreshed for each 24 h, and blastocyst development was evaluated on the 6th to 9th days.

Evaluation of meiotic resumption and cytoplasm maturation of oocytes

The cumulus cells were removed after the maturation culture of the cumulus–oocyte complex (COCs). The obtained oocytes were fixed in fixative solution for 24 h, and then stained with 1% resorcin blue solution for microscope (Nikon, Japan) examination. GVBD was used as the marker of meiotic resumption. The quality of cytoplasmic maturation was evaluated by the developmental ability of fertilized embryos and the freeze-resistant ability of oocytes.

Experimental design

Effects of milrinone on meiotic resumption efficiency of buffalo oocytes

To investigate the effect of milrinone on buffalo oocytes' nucleus maturation, the following three experimental treatments were performed on buffalo oocyte COC. First, buffalo oocytes ($n = 324$) were randomly divided into five groups and cultured for 24 h in TCM-199 mature medium containing milrinone at different concentrations (0, 12, 25, 50, 100 mol/l). Then the oocytes were fixed and stained, and GVBD was observed under microscope examination. Second, buffalo oocytes ($n = 865$) were cultured in a maturation medium containing 50 mol/l milrinone at different time intervals (0, 4, 8, 12, 16, 22, 24 h), and the oocytes were fixed and stained, and GVBD was observed under microscope examination. Third, buffalo oocytes ($n = 1186$) were randomly divided into three groups and cultured in basic maturation solution, basic maturation solution with 0.1 g/ml FSH and basic maturation solution with 0.1 g/ml FSH and 50 mol/l milrinone. Then the oocytes were fixed and stained, and GVBD were observed under microscopic examination.

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Effects of milrinone on buffalo oocytes cytoplasmic maturation

Buffalo oocytes ($n = 649$) were cultured in maturation solution with 50 mol/l milrinone for 16 h, and then further cultured in mature solution for 8 h, 12 h, 16 h and 20 h. Oocytes of normal morphology were used for fertilization. The cleavage rate of oocytes and blastocyst development rates were compared between different groups.

Data analysis

All the experimental data were analyzed using SAS 9.0 statistical software (SAS, Cary, NC, EUA) to determine the significance of the differences, and a P-value < 0.05 was considered as a significant difference.

Results

Effects of milrinone concentrations on meiotic resumption efficiency of buffalo oocytes

As shown in Figure 1, buffalo oocytes were cultured for 24 h in TCM-199 medium containing milrinone at different concentrations (0, 12, 25, 50, 100 mol/l). Milrinone (25–100 mol/l) inhibited the spontaneous maturation of buffalo COCs in a dose-dependent manner, also shown in the figure. In the 25, 50 and 100 mol/l milrinone groups, the GVBD rates (37.83 ± 6.46%, 25.93 ± 0.93% and 12.22 ± 5.43%) were significantly decreased compared with that of the control group (87.96 ± 4.05%) ($P < 0.01$). *In vitro* maturation of buffalo oocytes results are shown in Figure S1-1 to S1-6.

Effects of milrinone culture time interval to buffalo COCs meiotic resumption

In total, 865 COCs were tested in this investigation. The GV rates of a 50 mol/l milrinone culture for 4 h, 8 h, 12 h, 16 h, 22 h and 24 h were 95.83 ± 4.16%, 89.6 ± 5.79%, 86.11 ± 10.01%, 95.00 ± 5.00%, 82.8 ± 4.45% and 92.13 ± 3.96%, respectively, significantly higher than the control group of 79.03 ± 1.32%, 29.25 ± 2.41%, 19.60 ± 2.80%, 7.80 ± 1.13%, 5.75 ± 6.31% and 5.37 ± 5.95%, respectively ($P < 0.05$). Therefore, the inhibitory effect of milrinone on the maturation of buffalo COCs did not decrease with time.

Effects of milrinone on FSH-induced buffalo oocyte meiotic resumption

In total, 1186 COCs were used in this investigation. The GVBD rates of oocytes cultured in the mature medium supplemented with FSH and milrinone for different times (0, 4, 8, 12, 16, 22 and 24 h) were 10.07 ± 0.25%, 14.39 ± 7.46%, 12.29 ± 2.27%, 19.91 ± 9.36%, 54.17 ± 7.12%, 51.80 ± 7.17% and 57.67 ± 5.62%, respectively. At different mature culture times (8, 12, 16, 22 and 24 h) the GVBD rate of the control group with FSH was significantly increased compared with that of the FSH-added milrinone group (50.45% vs 12.29%, 81.35% vs19.91%, 86.90 % vs 54.17%, 87.27% vs 51.8% and 93.8% vs 57.67%, $P < 0.01$).

Effect of milrinone treatment on fertilization ability of buffalo oocytes

In total, buffalo oocytes ($n = 649$) were cultured in maturation solution with 50 mol/l milrinone for 16 h, and then further cultured in basic mature solution for another 8 h, 12 h, 16 h or 20 h. The matured oocytes were fertilized, the cleavage rate of 12 h, 16 h and 20 h groups were 59.1%, 48.5% and 50.6%, respectively, which

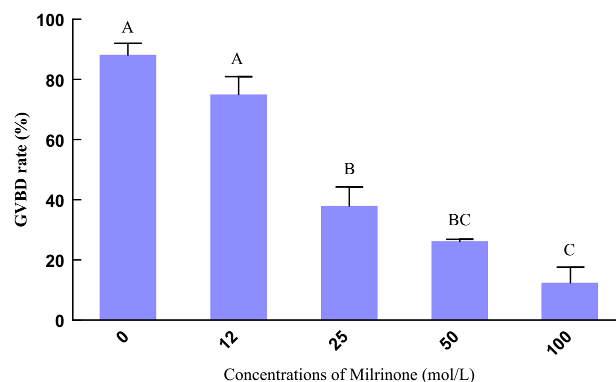

Figure 1. Effects of different concentrations of milrinone (0, 12, 25, 50, 100 mol/l) on the spontaneous maturation of buffalo COCs.

was significantly higher than that of the oocytes cultured for 8 h group at 29.5% ($P < 0.01$). Further blastocyst development for the 12 h, 16 h and 20 h groups was 36.9%, 33.6% and 23.9%, respectively, which was higher than for the 8 h group at 15.5%, but not different from that of milrinone free control group (33.4%) (please refer to Table 1). *In vitro* fertilization of buffalo oocytes results are shown in Figure S2-1 to S2-3.

Discussion

Effect of PDE inhibitor on buffalo oocytes meiotic resumption

In the present study, the *in vitro* maturation and *in vitro* fertilization (IVM–IVF) of oocytes made great progress, but the quality of the *in vitro* maturation oocytes was less than that of the *in vivo* mature oocytes (Sirard and Blondin, 1996). Most oocytes used in studies on IVF, nuclear transplantation and transgenic cloning are derived from *in vitro*-matured oocytes (Keefer, 2015; Lee and Maalouf, 2015; Silva *et al.*, 2018). At this time, the occurrence of GVBD and the extrusion of the first polar body are generally regarded as the criteria for oocytes maturation. The anisotropy between the maturation of the oocyte nucleus and cytoplasm is the main factor that affects the maturation of oocytes and subsequently embryonic development. Therefore, many researchers (Rime *et al.*, 1989; Yi and Park, 2005) have used a variety of phosphorylation inhibitors (such as cycloheximide, 6-DMAP) or cyclin-dependent protein kinase inhibitors (such as butyrolactone I, roscovitine) (Lonergan *et al.*, 2000; Le Beux *et al.*, 2003) to delay spontaneous maturation of the oocyte nucleus in *in vitro* maturation and extend cytoplasmic maturation time, therefore improving cytoplasmic maturation. Oocytes in the GV phase in the antral follicle can also resume meiosis when transferred from the follicular fluid into a hormone-free medium, this phenomenon is known as spontaneous maturation. Oocyte spontaneous maturation showed that the follicular fluid contains substances that can inhibit oocyte maturation such as hypoxanthine (HX), dcAMP and specific phosphodiesterase (PDE) inhibitors (Downs and Eppig, 1984; Downs *et al.*, 1985; Guimarães *et al.*, 2016). Milrinone, an inhibitor of phosphodiesterase that has been widely studied in recent years, can prevent the degradation of cAMP by inhibiting the activity of PDE, thereby increasing the level of cAMP in oocytes and maintaining oocyte maturation cleavage in the resting state (Eppig *et al.*, 1985; Downs *et al.*, 1989; Byskov *et al.*, 1997). Phosphodiesterase inhibitors inhibit the spontaneous recovery of oocyte meiosis by inhibiting the degradation of cAMP in the cytoplasm of oocytes.

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Table 1. Effect culture time on development of embryos derived from fertilized oocytes after matured in the maturation medium supplement with or without milrinone

Culture time in mature solution with milrinone (h)	Culture time in mature solution (h)	Fertilization number (N)	Cleavage rate (%)	Blastocyst rate (%)
0	24	132	89(66.2)[a]	31(33.4)[a]
16	8	103	27(29.5)[b]	4(15.5)[b]
16	12	122	71(59.1)[a]	26(36.9)[a]
16	16	165	80(48.5)[a]	21(33.6)[a]
16	20	127	65(50.6)[a]	15(23.9)[a,b]

[a,b,c]Different superscripts indicate significant difference among treatments ($P < 0.05$).

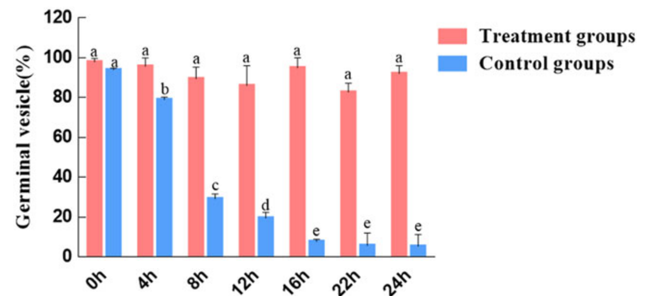

Figure 2. Effects of milrinone culture time intervals (0, 4, 8, 12, 16, 22 and 24 h) on buffalo COC meiotic resumption.

In this study, milrinone was used to inhibit the nuclear maturation of buffalo oocytes to improve the quality of oocyte maturation *in vitro*, to achieve the goal of nuclear and cytoplasmic synchronization. The results showed that milrinone (25–100 M) had a strong inhibitory effect on the spontaneous maturation of the buffalo oocyte complex *in vitro*, and the GVBD rate decreased with the increase in the milrinone dose, and showing drug dependency (Figure 1). Theoretically, this can be used as an inhibitor of nuclear maturation of buffalo oocytes maturation to improve the maturation quality of oocyte cytoplasm.

It has been reported that the addition of specific PDE inhibitors to bovine oocyte maturation solution can increase the level of cAMP in cumulus cells and oocytes and delay the process of meiosis, therefore increasing the ability of oocytes to support early embryonic development (Thomas *et al.*, 2002). Compared with rodents and primates, PDE inhibitors of ungulate animals have a short inhibitory time in GVBD (Thomas *et al.*, 2004). This is in contrast with the results of this experiment. The present study showed that the specific PDE inhibitor milrinone had a strong inhibitory effect on buffalo oocytes, and the inhibitory effect was not weakened within 24 h, also the process of meiosis was blocked (Figure 2). The influence of cAMP on substances required for GVBD, including protein synthesis or modification processes, may be different in different species. Conversely, hormones and growth factors in the medium are also affected by cAMP. This may be the reason why different researchers have reported different or even contradictory effects of cAMP or its analogues on oocyte maturation inhibition, especially in domestic animals. In addition to cAMP, there may be other oocyte maturation inhibitors, such as calcium, HX and IBMX. Oocyte maturation is also regulated by stimulating factors such as meiosis-activating alcohol (MAS) (Guo *et al.*, 2020). Whether the maturation of buffalo oocytes is influenced by these factors or by other factors has remained unrevealed and needs further study.

It is necessary to add FSH or forskolin, a type of adenylate cyclase activator, when maturation medium with milrinone is used to regulate spontaneous maturation of oocytes (Thomas *et al.*, 2004). FSH can activate adenylate cyclase in the oocyte to increase the level of cAMP in the oocyte, and then enter the oocyte through the interstitial connection between the oocytes, resulting in increased oocyte cAMP levels (Thomas *et al.*, 2002). It has also been reported that the addition of any particular type of PDE inhibitor, such as FSH, milrinone or rolipram, will slightly delay the initiation of the GVBD in the COCs. The process of meiosis was delayed, compared with oocytes that only had FSH added to the maturation solution (Thomas *et al.*, 2004). These results are consistent with our study. The addition of both milrinone and forskolin in the maturation solution maintained more COCs in the GV phase and delayed development to the MII phase compared with adding either drug (Thomas *et al.*, 2004). FSH promotes the generation of intracellular cAMP and delayed oocyte meiosis in cattle (Dominko *et al.*, 1998). Therefore, the nuclear maturation of oocytes was inhibited, the maturation time of oocytes was prolonged, finally the maturation quality of oocytes was improved. However, the mechanism by which PDE inhibitor can improve the quality of cytoplasmic maturation during *in vitro* maturation is still unclear.

In this study, the synergistic or antagonistic effects of FSH and milrinone on the inhibition of spontaneous maturation of buffalo oocytes cultured *in vitro* were studied (Figure 3). At the beginning of the maturation stage, both milrinone and FSH delayed meiosis recovery of the COCs, and they showed a synergistic effect. After 4 h, the inhibitory effect of milrinone could be overcome by adding FSH, which showed an antagonistic effect. Oocyte culture with milrinone but without FSH in the maturation medium was stopped at the GV phase after 24 h (Figure 2). However, the GVBD of oocytes cultured with FSH was delayed for 4 h. The main reason for this difference may be that milrinone has a strong inhibitory effect on the nuclear maturation of buffalo oocytes. The recovery of meiosis *in vivo* is consistent with increased gonadotropin levels. However, the mechanism by which gonadotropin promotes meiotic recovery is still unclear. Some studies have shown that the production of stimulating factors by granule cells/cumulus cells promotes meiotic recovery (Byskov *et al.*, 1997) confirmed that when hypoxanthine was added to the maturation medium, mouse COCs secreted substances that promoted meiosis after the FSH effect. This substance overcame the effects of meiosis inhibitors. Therefore, we need to investigate further whether there is a similar mechanism in buffalo oocytes to induce meiotic recovery.

Effect of PDE inhibitor on buffalo oocytes cytoplasm maturation

The quality of oocytes is an important factor affecting the success rate of nuclear transplantation and IVF. However, mammalian oocyte nuclear and cytoplasmic maturation are not synchronous. How to control the nuclear maturation of oocytes, prolong the maturation culture time, and enable the oocyte cytoplasm to fully mature have become the research focus of oocyte *in vitro* maturation research. The fertilization rate, embryo development ability and fetal birth rate are significantly improved when mouse and bovine oocytes are treated with phosphodiesterase inhibitors (Nogueira *et al.*, 2003; Thomas *et al.*, 2004). Milrinone is a

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Figure 3. Effects of milrinone on FSH-induced buffalo oocyte meiotic resumption.

phosphodiesterase inhibitor, which can prevent the degradation of cAMP by inhibiting the activity of PDE, thereby increasing the level of cAMP in oocytes and maintaining the state of oocyte maturation and cleavage (Eppig *et al*., 1985; Downs *et al*., 1989; Byskov *et al*., 1997). Thomas *et al.* (2004) added PDE3 inhibitor (milrinone, 100 mol/l) and PDE4 inhibitor (rolipram) into mature medium containing FSH, which significantly improved the blastocyst development rate of IVF after 28 h maturation culture and maturation cleavage. The effect of PDE inhibitor on *in vitro* maturation of buffalo oocytes has not been reported. Based on the work of Thomas *et al.* (2004), the present study also showed that milrinone had a strong inhibitory effect on the nuclear maturation of buffalo oocytes (Figure 2). At the same time, there was no significant difference between the inhibitory effect of milrinone when concentrations were at 50 mol/l and 100 mol/l (Figure 3), so the concentration of milrinone was reduced to 50 mol/l in the experiment. Oocytes cultured in mature medium with strong nuclear maturation inhibition need to be cultured in normal maturation medium for a time period to complete the final maturation stage. Nogueira *et al.* (2003) treated mouse oocytes with phosphodiesterase 3 inhibitor ORG 9935 (10 mol/l) for 24 h, and then cultured them for 18 h after normal maturation, which significantly improved the cleavage rate and morula development rate after IVF. Therefore, this study conducted a comparative experiment on the incubation time (8, 12, 16 and 20 h) of buffalo oocytes after culture for 16 h in normal maturation solution with milrinone. The results showed that the cleavage rate and blastocyst development rate of oocytes cultured for 12, 16 and 20 h were significantly higher than those cultured for 8 h. The reason may be the prolonged maturation culture time, as oocyte ageing reduces the developmental ability of oocytes. Conversely, whether the inhibition of nuclear maturation by inhibitors will affect the physiological function of organelles in the cytoplasm of buffalo needs to be explored further. Our results did not prove that milrinone could improve the *in vitro* maturation quality of buffalo oocytes, but it fully proved that milrinone had a reversible inhibition effect on nuclear maturation of buffalo oocytes, and that buffalo oocytes could mature normally after the inhibition was removed. Therefore, the reversible inhibitory effect of milrinone on oocyte maturation can be used to study the regulatory mechanism of oocyte maturation and its embryonic development potential.

In summary, milrinone inhibited the spontaneous maturation of buffalo COCs in a dose-dependent manner with the effective dose concentration of 25–100 M. The inhibitory effect of milrinone on spontaneous maturation of buffalo oocytes *in vitro* did not decrease with extension of the culture time, suggesting that milrinone can be used as a nuclear maturation inhibitor in the maturation process of buffalo oocytes. Milrinone significantly inhibited FSH-induced buffalo COC maturation *in vitro*, but this inhibition was partially overcome by FSH with time. The inhibition effect of milrinone on the nuclear maturation of buffalo oocytes was reversible. After the inhibition effect was removed, the buffalo oocytes could mature normally.

Supplementary material. For supplementary material accompanying this paper visit https://doi.org/10.1017/S0967199421000563

Supporting information. Supplementary figures are available for this paper online https://doi.org/10.1017/S0967199421000563

Author's contribution. DSS and WHX conceived and designed the experiments. GHB, WS and YGZ performed the experiments. LWD and WS drafted the manuscript. QYL and LS analyzed the data and revised the manuscript.

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Conflict of interest. The authors declare that no conflict of interest exists.

Ethical approval. Not applicable

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