

# Vitamin C protects porcine oocytes from microcystin-LR toxicity during maturation

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## Research

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# Abstract

**Background:** Microcystin-leucine arginine (MC-LR) is the most toxic cyanotoxin found in water bodies. Microcystins are produced as secondary products of cyanobacteria metabolism. They have a stable structure, and can bioaccumulate in living organisms. Humans and livestock who drink fresh water containing MC-LR can be poisoned. However, few studies have reported the effects of MC-LR exposure on livestock or human reproduction.

**Results:** We used porcine oocytes as the model to explore the effects of MC-LR on oocyte maturation, and studied the impact of vitamin C (VC) administration on MC-LR-induced meiosis defects. Exposure to MC-LR significantly restricted cumulus cell expansion and decreased first polar body extrusion. Further studies showed that MC-LR exposure led to meiosis arrest by disturbing cytoskeleton dynamics with MC-LR exposed oocytes displaying aberrant spindle organization, low levels of acetylate  $\alpha$ -tubulin, and disturbed actin polymerization. Additionally, MC-LR exposure impaired cytoplasmic maturation by disturbing mitochondria distribution. Moreover, MC-LR also produced abnormal epigenetic modifications, and induced high levels of oxidative stress and DNA damage. The administration of VC provided partial protection from all of the defects observed in oocytes exposed to MC-LR.

**Conclusions:** These results demonstrate that MC-LR has a toxic effect on oocyte meiosis through the generation of excessive ROS levels and DNA damage. Supplementation of VC was able to protect against MC-LR-induced oocyte damage and represents a potential therapeutic strategy to improve the quality of MC-LR-exposed oocytes.

## 1. Introduction

The incidence of cyanobacteria blooms is increasing globally due to water eutrophication issues and global warming, and such blooms are now recognized as an emerging environmental threat[1]. Cyanobacterial blooms generate a secondary metabolite that can be highly toxic. Microcystins (MCs) are the most abundant and common cyanotoxins produced by toxic cyanobacteria and there has been a marked increase in the reports of human and livestock poisonings from the consumption of fresh water containing MCs[2–4]. These cyanotoxins are characterized by their highly stable structure. They can bioaccumulate in aquatic animals owing to their ability to resist degradation under conditions found in most natural water ways (near-neutral pH) and are also resistant to high temperatures. [5] Microcystin-leucine-arginine (MC-LR) is one of the most abundant and harmful microcystins[6, 7]. It is a potent hepatotoxin that has been linked to the development of primary liver cancer and is classified as a potential human carcinogen (Group 2B) by the International Agency for Research on Cancer (IARC) [8–10]. A variety of toxicity tests have shown that MC-LR causes oxidative stress, and induces apoptosis and DNA damage, in mouse and human hepatocytes both in vitro and in vivo[11–14]. Accumulation of MC-LR has also been reported in reproductive organs, including the connective tissue of the ovary and the testis, as well as in oocytes[15], and can severely impair the function of the reproductive system. In the zebrafish, MC-LR affected the endocrine system and oogenesis, and disrupted the meiotic maturation of

oocytes in vitro[16–19]. MC-LR has also been reported to decrease sperm motility in male rats and induce toxic effects on Sertoli cells of the rat testis[20, 21]. However, the effects of MC-LR on human reproductive function have not yet been studied.

Vitamin C (L-ascorbic acid; VC) is a well-known antioxidant. Acting as a free radical scavenger, VC can donate electrons to reduce reactive oxygen species (ROS) and prevent damage to lipids, proteins and DNA during cell metabolism or from exposure to toxins and pollutants[22–24]. Studies have proved that VC is beneficial for mammalian reproduction. It can improve the development of preantral follicles during in vitro culture[25, 26], promote the meiotic maturation of pig oocytes[27, 28], and improve the developmental competence of embryos after parthenogenetic activation and somatic cell nuclear transplantation[29]. Furthermore, VC can ameliorate defects caused by environmental pollutants such as gamma-irradiation and heavy metal pollution in oocytes and embryos[30, 31]. Therefore, we hypothesized that VC could protect oocytes from defects induced by MC-LR during maturation. Given that pigs share many physiological similarities with humans, and porcine cells are easier to obtain than human cells, we used the porcine oocyte as a model to investigate the toxic effects of MC-LR.

## 2. Materials And Methods

### 2.1. Antibodies and chemicals

Antibodies were as follows: Mouse monoclonal anti- $\alpha$ -tubulin antibody (1:200, Sigma, St. Louis, MO, USA, #F2168); anti-acetyl- $\alpha$ -tubulin (Lys-40) antibody (1:100, Sigma #T7451); phalloidin-TRITC (1:200, Sigma, #P1951); rabbit monoclonal to gamma H2A.X (gH2A.X) (Abcam, Cambridge, United Kingdom, #ab81299); rabbit polyclonal anti-di-methyl-histone H3 (Lys4) (H3K4me2) antibody (Cell Signaling Technology, Devers, MA, USA); Alexa Fluor 488 goat anti-mouse antibody (1:200, Invitrogen #A11126, Carlsbad, CA, USA); MitoTracker Red CMXRos (Thermo Fisher Scientific, #7512, Waltham, MA, USA); Alexa Fluor 594 goat anti-rabbit antibody (Invitrogen, Carlsbad, CA, United States). Microcystin-LR was purchased from APExBio Technology (Houston, USA, #B3698); VC was purchased from Sigma (Shanghai, China, #A7506). The basic maturation culture medium used was tissue culture medium (TCM-199; Sigma). Phosphate-buffered saline (PBS) was purchased from Life Technologies (Invitrogen).

### 2.2. Oocyte collection and in vitro maturation (IVM)

Ovaries were obtained from a local slaughterhouse and transported to the laboratory in 0.9% NaCl containing 800 IU/ml of gentamicin at 37 °C. In vitro oocyte maturation was performed as previously described[32]. Follicular fluid was collected from 3–8 mm follicles using an 18-gauge needle attached to a 10-ml disposable syringe. Cumulus–oocyte complexes (COCs) were then aspirated by vacuum suction from follicular fluid. After washing, the COCs with compact cumulus cells and a uniform ooplasm were selected to culture in 4-well dishes with in vitro maturation medium (IVM) (TCM-199 supplemented with 10% cattle serum (CS; Gibco), 10% (v/v) porcine follicular fluid, 0.8 mM l-glutamine, 75 mg/mL penicillin, 50 mg/mL streptomycin, 15 IU/mL pregnant mare serum gonadotropin (PMSG), and 15 IU/mL human chorionic gonadotropin (hCG)) at 38.5 °C in an atmosphere of 5% CO<sub>2</sub> with saturated humidity. After

further culture for 42–44 h, COCs were treated with hyaluronidase (1 mg/ml in TCM-199 culture medium) for approximately 1 min to obtain denuded oocytes (DOs).

## **2.3. MC-LR and VC treatment**

MC-LR was dissolved in TCM-199 culture medium and then in maturation medium to final concentrations of 20, 40, 80 and 120  $\mu\text{M}$ . VC was dissolved in PBS and diluted with maturation medium to final concentrations of 100  $\mu\text{M}$  and 500  $\mu\text{M}$ . The VC was added to maturation medium immediately before use.

## **2.4. Immunofluorescence staining and confocal microscopy**

Denuded oocytes were collected and fixed in 4% paraformaldehyde (PFA) in PBS for 30 min at room temperature. After being washed for 15 min in wash buffer ( $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ -free PBS with 1% BSA (PB1)), oocytes were permeabilized in 1% Triton X-100 (in PBS) for 1 h at room temperature. Subsequently, they were blocked with PB1 for 1 h at room temperature to suppress the non-specific binding of IgG. Denuded oocytes were then incubated with primary antibodies overnight at 4 °C, washed three more times, and incubated with secondary antibody for 1 h at room temperature. Finally, Hoechst 33342 (10  $\mu\text{g}/\text{mL}$ ) was used to stain nuclei for 10 min at room temperature. Samples were mounted on glass slides and examined with a confocal laser-scanning microscope (LSM 700 META; Zeiss, Oberkochen, Germany).

## **2.5. Detection of Mitochondria and Reactive Oxygen Species (ROS)**

A DCFH diacetate (DCFHDA) kit (Beyotime, China) was used to examine the level of intracellular ROS generated during oocyte maturation. Mito-Tracker Red CMXRos (Invitrogen, Eugene, OR, United States, #M7512,) was used for mitochondria detection. After DOs were obtained, they were incubated in TCM-199 culture medium containing DCFHDA (1:800) or Mito-Tracker Red CMXRos (1:200) for 30 min at 38.5 °C in a 5%  $\text{CO}_2$  incubator. After three washes in TCM-199, oocytes were placed on a glass slide and observed under the confocal laser-scanning microscope as soon as possible.

## **2.6. Statistical analysis**

At least three replicates were performed for each experiment. All analyses were performed using Graph Pad Prism 5 (Graph Pad Software Inc., San Diego, CA, USA) and were presented as mean percentages  $\pm$  standard error of the mean (mean  $\pm$  SEM). Inter-group differences were compared using t-tests.  $p$  values  $<$  0.05 indicated statistical significance.

# **3. Results**

## **3.1. Vitamin C reduces meiotic defects in MC-LR-exposed oocytes**

To explore the toxic effects of MC-LR during maturation oocytes were cultured with varying concentrations of MC-LR (0  $\mu$ M, 20  $\mu$ M, 40  $\mu$ M, 80  $\mu$ M, and 120  $\mu$ M) for 44 h in vitro. The proportion of polar body extrusion (PBE) and COC viability in the control and MC-LR-exposed oocytes is shown in Fig. 1. Almost all of the cumulus cells surrounding oocytes in the control group were fully expanded, while those in the MC-LR-exposed group exhibited poor expansion of COCs (Fig. 1A). Moreover, the majority of oocytes in the control group reached the meiosis II (MII) stage after 44 h of culture, and demonstrated extrusion of the polar body, but exposure to MC-LR significantly reduced the PBE rate (control:  $64.52 \pm 3.11\%$ ,  $n = 131$ ; 20  $\mu$ M:  $60.10 \pm 8.71\%$ ,  $n = 108$ ,  $p > 0.05$ ; 40  $\mu$ M:  $50.18 \pm 4.36\%$ ,  $n = 153$ ,  $p < 0.05$ ; 80  $\mu$ M:  $39.15 \pm 5.39\%$ ,  $n = 152$ ,  $p < 0.01$ ; 120  $\mu$ M:  $29.63 \pm 2.32\%$ ,  $n = 148$ ,  $p < 0.001$ ; Fig. 1B). The concentration of 80  $\mu$ M MC-LR was chosen for further studies because this not only caused obvious meiotic defects, but also allowed a proportion of oocytes to develop to the MII stage for further investigation.

To investigate whether VC can alleviate meiotic arrest caused by MC-LR, VC was supplemented to the IVM culture medium containing 80  $\mu$ M MC-LR. We found that 100  $\mu$ M VC significantly increased the rate of PBE in MC-LR exposure oocytes compared with MC-LR alone ( $61.21 \pm 2.17\%$ ,  $n = 150$ , VS  $41.42 \pm 4.7\%$ ,  $n = 145$ ,  $p < 0.01$ ) but the higher concentration of 500  $\mu$ M VC did not show the same effect ( $39.93 \pm 2.68\%$ ,  $n = 115$ ,  $p > 0.05$ ) (Fig. 1A and C). Thus, the concentration of 100  $\mu$ M VC was chosen for further study. These results suggested that MC-LR exposure inhibited porcine oocyte maturation in a dose-dependent manner, but VC can protect oocytes against meiotic defects caused by MC-LR exposure.

Figure 1. Vitamin C (100  $\mu$ M) alleviates the meiotic defects in MC-LR-exposed oocytes. (A) Representative images of cumulus expansion and polar body extrusion (PBE) in the control, MC-LR-exposed and VC-rescued groups. Bar = 150  $\mu$ m (i); 100  $\mu$ m (ii); 20  $\mu$ m (iii). (B) The rate of PBE was compared in control and different concentrations of MC-LR-exposed groups (20  $\mu$ M, 40  $\mu$ M, 80  $\mu$ M and 120  $\mu$ M) after being cultured for 44 h in vitro. (C) The rate of PBE was recorded in control and different concentrations of VC-supplemented groups (100  $\mu$ M and 500  $\mu$ M) after culture for 44 h with 80  $\mu$ M MC-LR in vitro. ns: no significant difference, \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

## 3.2. VC alleviates spindle defects in MC-LR-exposed oocytes

Given that spindle formation is critical for PBE, we next examined spindle dynamics after MC-LR exposure. The results of immunofluorescence are shown in Fig. 2. Most oocytes in the control group exhibited regular spindle morphology and good chromosome alignment on the equatorial plate. In contrast, spindle formation was severely disrupted, and the chromosomes were disorganized, in the MC-LR-exposed group. Quantitative analysis showed that MC-LR-exposed oocytes exhibited a significantly higher proportion of aberrant spindles than control oocytes ( $77.58 \pm 7.23\%$ ,  $n = 116$ , VS  $16.57 \pm 4.05\%$ ,  $n = 106$ ,  $p < 0.01$ ) (Fig. 2B). However, supplementation of VC decreased the proportion of abnormal spindles caused by MC-LR exposure ( $35.27 \pm 4.05\%$ ,  $n = 104$ , VS  $77.58 \pm 7.23\%$ ,  $n = 116$ ,  $p < 0.01$ ), indicating that VC can restore spindle defects in MC-LR-exposed oocytes.

Figure 2. Vitamin C protects MC-LR-exposed oocytes from spindle defects. (A) Representative images of spindle morphology and chromosome alignment in the control, MC-LR-exposed and VC-rescued groups. Bar = 5  $\mu\text{m}$ . (B) The rate of aberrant spindles in the control, MC-LR-exposed and VC-rescued groups.  $**p < 0.01$ .

### 3.3. VC restores $\alpha$ -tubulin acetylation level in MC-LR-exposed oocytes

We next examined the level of acetylated  $\alpha$ -tubulin, through immunofluorescence, because this post-translational modification is critical for the maintenance of stable microtubules in both mitotic and meiotic cells. As shown in Fig. 3, acetylated tubulin levels were significantly lower in MC-LR-exposed oocytes compared with control oocytes. Furthermore, VC supplementation significantly increased the level of acetylated  $\alpha$ -tubulin in MC-LR-exposed oocytes. Quantitative analysis of the fluorescence intensity of acetylated  $\alpha$ -tubulin validated these qualitative findings (MC-LR-exposed:  $4.29 \pm 0.79$ ,  $n = 60$ , VS Control:  $15.60 \pm 1.71$ ,  $n = 60$ ,  $p < 0.001$ ; VC supplementation:  $8.64 \pm 0.92$ ,  $n = 60$ , VS MC-LR-exposed:  $4.29 \pm 0.79$ ,  $n = 60$ ,  $p < 0.01$ ). (Fig. 3B). These results suggest that MC-LR may disorder spindle assembly by downregulating tubulin acetylation and that the presence of vitamin C can partly prevent these aberrations in oocyte development.

Figure 3. Vitamin C increases the level of acetylation of  $\alpha$ -tubulin in MC-LR-exposed oocytes. (A) Representative images of  $\alpha$ -tubulin in the control, MC-LR-exposed and VC-rescued groups. Bar = 5  $\mu\text{m}$ . (B) Quantitative analysis of the fluorescence intensity of acetylated  $\alpha$ -tubulin in the control, MC-LR-exposed and VC-rescued groups.  $**p < 0.01$ ,  $***p < 0.001$ .

### 3.4. Vitamin C improves actin dynamics of MC-LR-exposed oocytes

Because actin filaments are the main driving force for asymmetric division in mammalian oocytes, we next examined the actin dynamics in both control oocytes and MC-LR-exposed oocytes. Phalloidin was used to label F-actin, and the results are shown in Fig. 4. In the control group, the actin filaments in most oocytes were evenly distributed on the plasma membrane and showed a strong immunofluorescent signal. However, in most MC-LR-exposed oocytes, the actin signal was so significantly reduced it was almost undetectable (Fig. 4A and B). Quantitative analysis of the actin fluorescence intensity (Fig. 4C) also showed a significant decrease in MC-LR-exposed oocytes, compared with the control group ( $1.26 \pm 0.34$ ,  $n = 60$ , VS  $60.24 \pm 8.20$ ,  $n = 60$ ,  $p < 0.001$ ) and this was partially ameliorated by the co-supplementation of VC ( $20.30 \pm 3.74$ ,  $n = 60$ ) (Fig. 4A and C). Moreover, the proportion of mislocalized actin was significantly increased in MC-LR-exposed oocytes (MC-LR-exposed:  $79.58 \pm 2.00\%$ ,  $n = 96$ , VS Control:  $22.93 \pm 4.34\%$ ,  $n = 100$ ,  $p < 0.001$ ) and co-supplementation with VC supplementation significantly reduced actin abnormalities caused by MC-LR exposure (VC supplement:  $49.27 \pm 3.59\%$ ,  $n = 110$ , VS MC-LR-exposed:  $79.58 \pm 2.00\%$ ,  $n = 96$ ,  $p < 0.01$ ) (Fig. 4A and B). These results show that VC is able to partially protect porcine oocytes from actin damage caused by MC-LR exposure.

Figure 4. Vitamin C improves the actin dynamics of MC-LR-exposed oocytes. (A) Representative images of actin distribution in the control, MC-LR-exposed and VC-rescued groups. Bar = 20  $\mu\text{m}$ . (B) The rate of mislocalization of actin. (C) Quantitative analysis of the fluorescence intensity of actin in the control, MC-LR-exposed and VC-rescued groups.  $**p < 0.01$ ,  $***p < 0.001$ .

### 3.5. Vitamin C reduces mitochondrial abnormalities in MC-LR-exposed oocytes

Mitochondria are essential for oocyte maturation. Abnormal mitochondria lead to a decrease in oocyte quality and prevent embryonic development[33]. To determine whether MC-LR exposure caused abnormal mitochondria, we used MitoTracker Red CMXRos to label mitochondria, and the results are shown in Fig. 5. In control oocytes, the mitochondria signals were mainly seen in the subcortical regions around lipid droplets. The exposure of oocytes to MC-LR resulted in an abnormal pattern of mitochondrial distribution (Fig. 5A). Quantitative fluorescence intensity analysis showed that the mitochondrial signals were reduced in MC-LR-exposed oocytes compared with the control group ( $7.18 \pm 0.74$ ,  $n = 60$ , VS  $19.06 \pm 0.98$ ,  $n = 60$ ,  $p < 0.001$ ) (Fig. 5B). Supplementation of VC to MC-LR-exposed oocytes caused the distribution of mitochondria in these samples to appear similar to the control group and increased the fluorescent intensity of the mitochondrial signal compared with MC-LR-exposed oocytes which did not receive VC supplementation ( $11.50 \pm 0.80$ ,  $n = 60$ , VS  $7.18 \pm 0.74$ ,  $n = 60$ ,  $p < 0.001$ ) (Fig. 5). These results suggest that VC can protect oocytes from mitochondria damage caused by MC-LR exposure.

Figure 5. Vitamin C protects MC-LR-exposed oocytes from mitochondrial damage. (A) Representative images of mitochondria distribution in the control, MC-LR-exposed and VC-rescued groups. Bar = 20  $\mu\text{m}$ . (B) Quantitative analysis of the fluorescence intensity of mitochondria.  $***p < 0.001$ .

### 3.6. Vitamin C restores abnormal epigenetic alterations in MC-LR-exposed oocytes

Histone methylation modification is a pivotal epigenetic modification that is critical for the regulation of gene expression and gene silencing. Disruption of histone modifications in the oocyte can lead to meiotic arrest[[34]. The level of histone H3 lysine 4 di-methylation (H3K4me2), which is associated with active transcription, was studied to assess potential epigenetic modifications in MC-LR-exposed oocytes. As shown in Fig. 6, the fluorescence intensities of H3K4me2 were significantly reduced in MC-LR-exposed oocytes compared with the control group. However, VC supplementation alleviated this defect to some extent (Fig. 6A). Quantitative analysis also confirmed this (MC-LR-exposed:  $6.012 \pm 0.69$ ,  $n = 60$ , VS Control:  $21.53 \pm 0.94$ ,  $n = 60$ ,  $p < 0.001$ ; VC supplement:  $16.14 \pm 1.61$ ,  $n = 60$ , VS MC-LR-exposed:  $6.012 \pm 0.69$ ,  $n = 60$ ,  $p < 0.001$ ). (Fig. 6B) These results demonstrate that VC can protect oocytes from abnormal epigenetic alterations caused by MC-LR exposure.

Figure 6. Vitamin C can protect oocytes from abnormal epigenetic alterations caused by MC-LR exposure. (A) Representative images of H3K4me2 in the control, MC-LR-exposed, and VC-rescued groups. Oocytes were stained with rabbit polyclonal anti-H3K4me2 antibody to visualize H3K4me2 (green) and counterstained with Hoechst 33342 to visualize chromosomes (blue). Bar = 5  $\mu$ m. (B) Quantitative analysis of the fluorescence intensity of H3K4me2. \*\*\* $p < 0.001$ .

### 3.7. VC decreases oxidative stress and alleviates DNA damage in MC-LR -exposed oocytes

MC-LR induces cytotoxicity via oxidative stress in many kinds of cells, including tissues of the ovary[20, 35]. To investigate whether MC-LR was inhibiting oocyte maturation via oxidative stress, we used DCFH staining to compare the ROS levels between control and MC-LR-exposed oocytes. MC-LR exposure resulted in increased ROS levels compared with oocytes in the control group ( $42.06 \pm 5.09$ ,  $n = 60$ , VS  $2.73 \pm 0.46$ ,  $n = 60$ ,  $p < 0.001$ ; Fig. 7A) and VC significantly reduced the excessive ROS present in MC-LR-exposed oocytes ( $10.28 \pm 1.16$ ,  $n = 60$ , VS  $42.06 \pm 5.09$ ,  $n = 60$ ,  $p < 0.001$ ) (Fig. 7A and B). These results suggest that VC decreased oxidative stress caused by MC-LR exposure during oocyte maturation.

Because oxidative stress can damage DNA, and MC-LR has been reported to inhibit DNA repair[36], we examined DNA damage by  $\gamma$ -H2A.X staining. Higher levels of  $\gamma$ -H2A.X signal were found in MC-LR-exposed oocytes compared with the control group, whereas VC supplement significantly reduced the  $\gamma$ -H2A.X signal (MC-LR-exposed:  $32.21 \pm 2.84$ ,  $n = 60$ , VS Control:  $5.38 \pm 0.66$ ,  $n = 60$ ,  $p < 0.001$ ; VC supplement:  $12.53 \pm 0.81$ ,  $n = 60$ , VS MC-LR-exposed:  $32.21 \pm 2.84$ ,  $n = 60$ ,  $p < 0.001$ ; Fig. 7C and D). These results suggest that VC can protect oocytes from DNA damage caused by MC-LR exposure.

Figure 7. VC decreases oxidative stress and alleviates DNA damage in MC-LR -exposed oocytes. (A) Representative images of ROS level in the control, MC-LR-exposed and VC-rescued groups. Bar = 20  $\mu$ m. (B) Quantitative analysis of the fluorescence intensity of ROS. \*\*\* $p < 0.001$ . (C) Immunofluorescent staining of  $\gamma$ H2A.X showing DNA damage in control, MC-LR-exposed and VC-rescued groups. Bar = 5  $\mu$ m. (D) Quantitative analysis of the fluorescence intensity of  $\gamma$ H2A.X. \*\*\* $p < 0.001$ .

## 4. Discussion

The United States, China, Japan, and Europe have reported MC-LR contamination of freshwater resources and the frequency of these reports is increasing worldwide[37]. The cyanotoxin can bioaccumulate in living organisms and its chemical structure is highly stable during cooking, and resistant to chemical breakdown[5]. Humans and livestock living near freshwater bodies contaminated with cyanobacteria can be poisoned by drinking water containing MC-LR. However, the current studies on MC-LR reproductive toxicity are predominately focused on fish. Few studies have reported the effects on mammalian reproduction, including that of humans. To investigate the effects of MC-LR on mammalian reproductive function, we used porcine oocytes as a model because of their similar physiology to human oocytes. Our

results showed that appropriate doses of VC were able to protect against abnormalities to cytoskeleton dynamics, mitochondrial distribution, epigenetic modification, and oxidative stress caused by MC-LR.

Given that cumulus cell expansion and the first PBE rate are two critical biological events occurring during meiotic progression[38], we first examined these two important events and found poor expansion of COCs and a depressed PBE rate in MC-LR-exposed oocytes. This indicated that oocyte maturation was retarded by MC-LR exposure in a dose-dependent manner. The supplementation of VC with MC-LR partially protected oocytes from the negative effect of MC-LR on COC expansion and PBE during oocyte maturation. To further investigate how MC-LR exposure causes meiosis failure we examined cytoskeleton dynamics because they are essential for cell cycle progression and polar body extrusion during oocyte maturation. The results showed that spindle morphology and actin distribution in oocytes were severely disrupted by MC-LR. Moreover, the acetylation level of  $\alpha$ -tubulin, which is a marker of spindle stability[39], was significantly reduced. These results are also consistent with previous studies that MC-LR exposure induced microfilament and microtubule alterations, and caused the progressive disassembly of actin fibers in many kinds of cells[40–42]. However, in VC-rescued oocytes, the cytoskeleton morphology and stability were restored to some extent. These results suggest that abnormalities in the cytoskeleton dynamics from MC-LR exposure lead to meiotic arrest in the porcine oocytes, and these effects can be partly mitigated by the supplementation of VC.

The mitochondrion is a primary organelle that supplies the majority of the cellular ATP for oocyte maturation. Mitochondrial integrity is therefore considered essential for oocyte cytoplasmic maturation [43, 44]. To further study the causes of oocyte meiotic arrest caused by MC-LR, we investigated mitochondrial distribution in MC-LR-exposed and VC-rescued oocytes. Considering that changes in ATP levels correlate with the distribution of mitochondria present during oocyte maturation [45], the abnormal activity of mitochondria in MC-LR-exposed oocytes may also explain the failure of meiosis caused by MC-LR exposure. For this parameter, VC supplementation was also effective in ameliorating mitochondrial abnormalities caused by MC-LR exposure.

Besides cytoskeleton dynamics and mitochondrion distribution, epigenetics programming is also a critical event for oocyte maturation[34]. Histone modifications are pivotal epigenetic modifications that widely regulate gene transcription expression and gene silencing. Disruption of histone modifications causes defective chromosome condensation and segregation, delayed maturation progression and even oocyte aging [46, 47]. In this study, H3K4me2, an activating epigenetic marker[47, 48], was reduced by MC-LR exposure, indicating that MC-LR meiotic maturation failure may result from an impairment of epigenetic modifications in porcine oocytes. However, VC restored these abnormal epigenetic alterations in MC-LR-exposed oocytes.

Several studies have indicated that MC-LR increases ROS and inhibits the repair of damaged DNA damage in HepG2 and human lymphocyte cell lines[11, 36, 49]. We hypothesized that MC-LR-induced oocyte meiosis arrest may be caused by exposure to high ROS levels and DNA damage. Increased levels of ROS have previously been associated with cytoskeletal disorganization and cell cycle arrest in human

oocytes and high levels of DNA damage have been shown to cause oocyte arrest at meiosis I (MI) [50, 51]. Therefore, it is likely that the high levels of ROS found in our study, and the associated DNA damage, contributed to oocyte arrest in the MC-LR-exposed treatment group. But vitamin C supplementation in MC-LR-exposed oocytes reduced ROS levels in MC-LR-exposed oocytes.

## 5. Conclusion

Taken together, our results indicate that the presence of MC-LR is deleterious to the maturation of porcine oocytes. The cyanotoxin generates excessive intracellular ROS levels and causes DNA damage. Supplementation with vitamin C can reduce the severity of MC-LR-induced cell defects and provides a potential therapeutic strategy to improve the quality of MC-LR-exposed oocytes.

## Declarations

### Author contributions

All authors were involved in designing and planning experiments, preparing and reviewing the article. Xue Zhang, Changyin Zhou, Weijian Li, performed the experiments; Xue Zhang and Honglin Liu. Analysed the data; Xue Zhang and Honglin Liu wrote the article.

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### Availability of data and materials

All data generated or analyzed during this study are available from the corresponding author on reasonable request.

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Conflict of interest

The authors declare that there is no conflict of interests.

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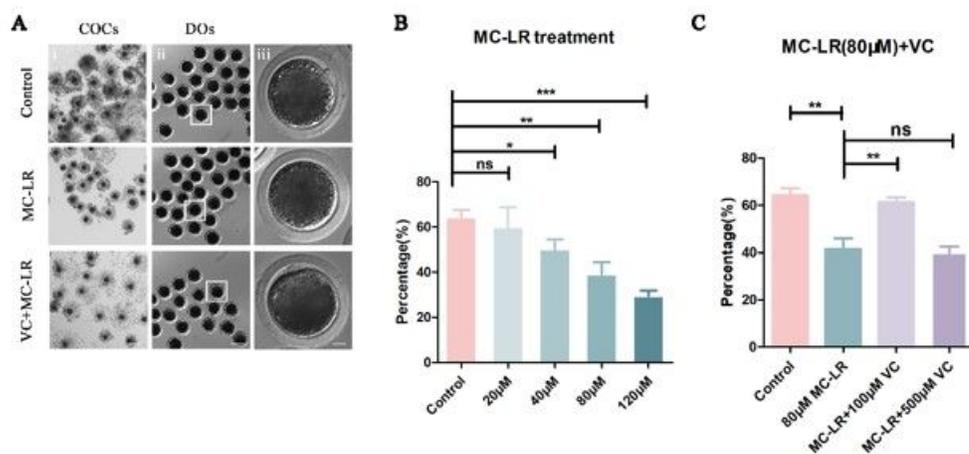
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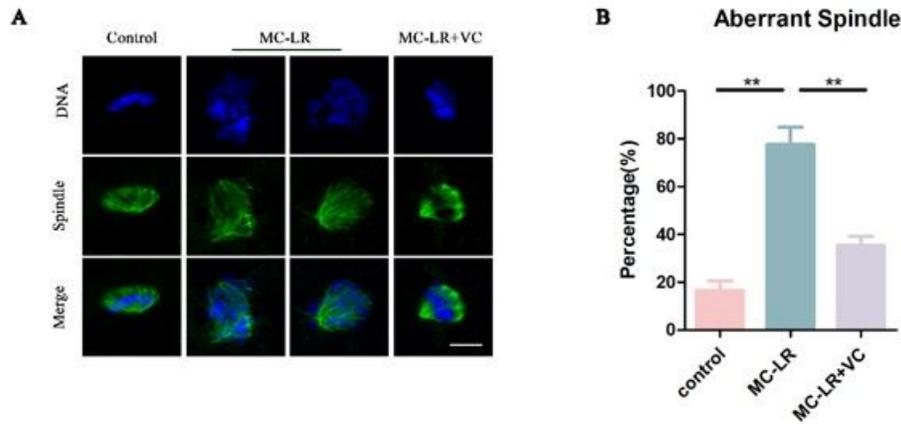
## Figures



**Figure 1**

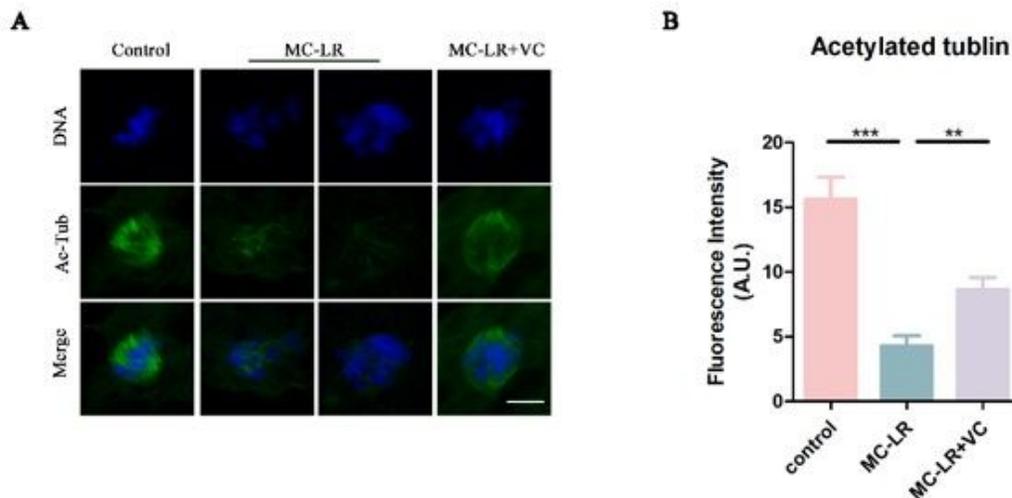
Vitamin C (100 µM) alleviates the meiotic defects in MC-LR-exposed oocytes. (A) Representative images of cumulus expansion and polar body extrusion (PBE) in the control, MC-LR-exposed and VC-rescued groups. Bar=150 µm (i); 100 µm (ii); 20 µm (iii). (B) The rate of PBE was compared in control and different concentrations of MC-LR-exposed groups (20 µM, 40 µM, 80 µM and 120 µM) after being cultured for 44

h in vitro. (C) The rate of PBE was recorded in control and different concentrations of VC-supplemented groups (100  $\mu$ M and 500  $\mu$ M) after culture for 44 h with 80  $\mu$ M MC-LR in vitro. ns: no significant difference, \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .



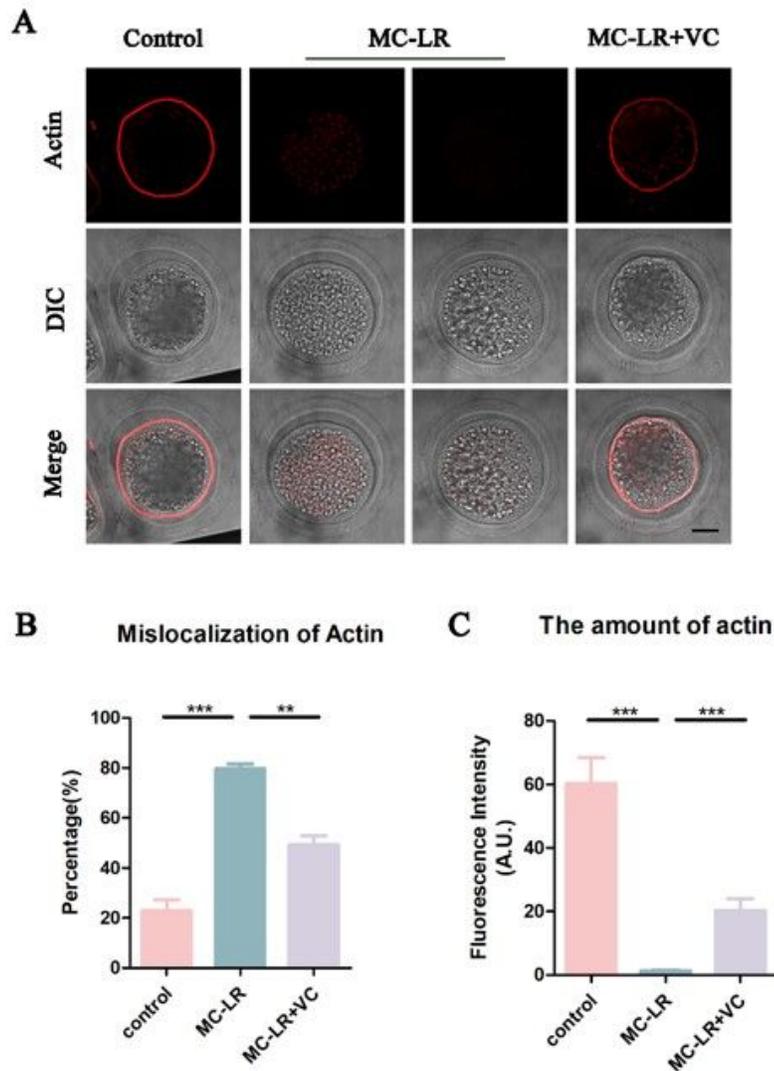
**Figure 2**

Vitamin C protects MC-LR-exposed oocytes from spindle defects. (A) Representative images of spindle morphology and chromosome alignment in the control, MC-LR-exposed and VC-rescued groups. Bar = 5  $\mu$ m. (B) The rate of aberrant spindles in the control, MC-LR-exposed and VC-rescued groups. \*\* $p < 0.01$ .



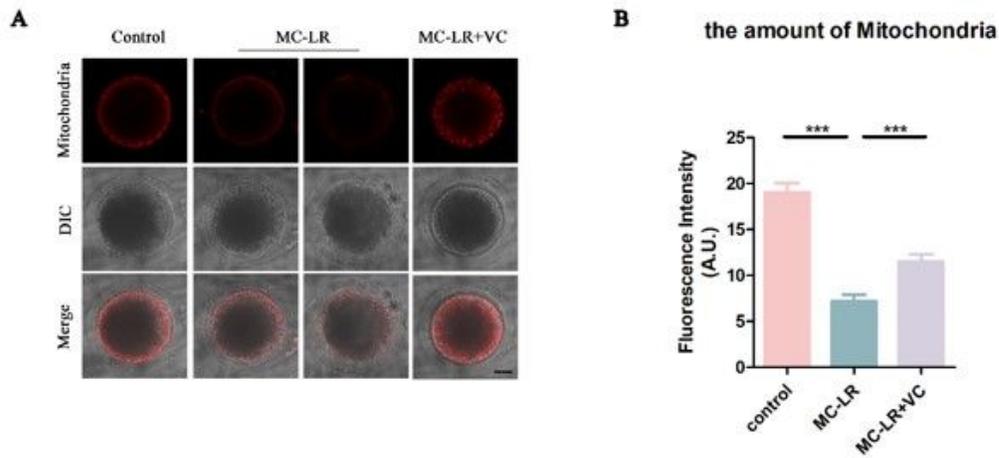
**Figure 3**

Vitamin C increases the level of acetylation of  $\alpha$ -tubulin in MC-LR-exposed oocytes. (A) Representative images of  $\alpha$ -tubulin in the control, MC-LR-exposed and VC-rescued groups. Bar=5  $\mu$ m. (B) Quantitative analysis of the fluorescence intensity of acetylated  $\alpha$ -tubulin in the control, MC-LR-exposed and VC-rescued groups. \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .



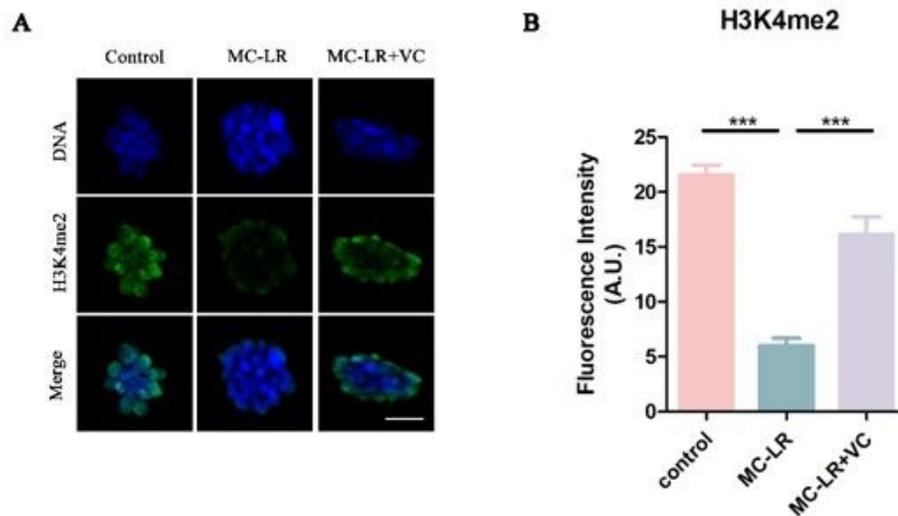
**Figure 4**

Vitamin C improves the actin dynamics of MC-LR-exposed oocytes. (A) Representative images of actin distribution in the control, MC-LR-exposed and VC-rescued groups. Bar=20  $\mu$ m. (B) The rate of mislocalization of actin. (C) Quantitative analysis of the fluorescence intensity of actin in the control, MC-LR-exposed and VC-rescued groups. \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .



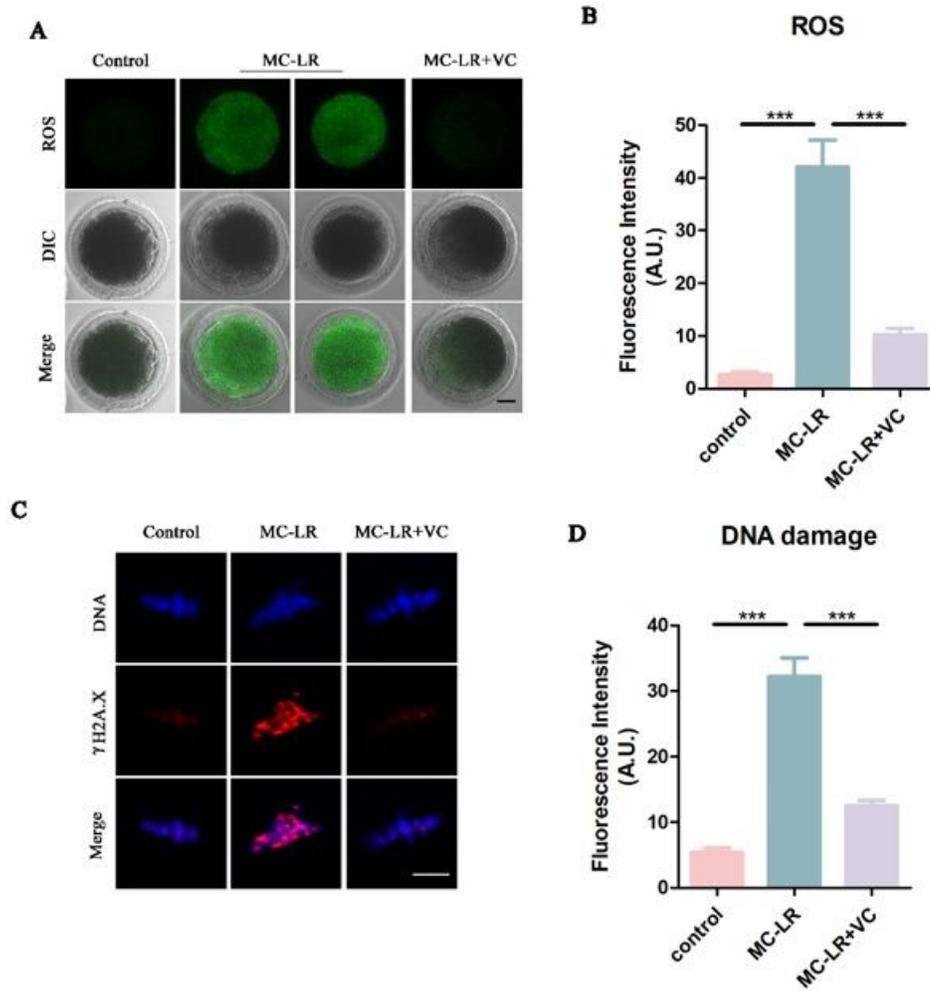
**Figure 5**

Vitamin C protects MC-LR-exposed oocytes from mitochondrial damage. (A) Representative images of mitochondria distribution in the control, MC-LR-exposed and VC-rescued groups. Bar=20  $\mu$ m. (B) Quantitative analysis of the fluorescence intensity of mitochondria. \*\*\* $p < 0.001$ .



**Figure 6**

Vitamin C can protect oocytes from abnormal epigenetic alterations caused by MC-LR exposure. (A) Representative images of H3K4me2 in the control, MC-LR-exposed, and VC-rescued groups. Oocytes were stained with rabbit polyclonal anti-H3K4me2 antibody to visualize H3K4me2 (green) and counterstained with Hoechst 33342 to visualize chromosomes (blue). Bar=5  $\mu$ m. (B) Quantitative analysis of the fluorescence intensity of H3K4me2. \*\*\* $p < 0.001$ .



**Figure 7**

VC decreases oxidative stress and alleviates DNA damage in MC-LR -exposed oocytes. (A) Representative images of ROS level in the control, MC-LR-exposed and VC-rescued groups. Bar=20  $\mu$ m. (B) Quantitative analysis of the fluorescence intensity of ROS. \*\*\* $p < 0.001$ . (C) Immunofluorescent staining of  $\gamma$ H2A.X showing DNA damage in control, MC-LR-exposed and VC-rescued groups. Bar=5  $\mu$ m. (D) Quantitative analysis of the fluorescence intensity of  $\gamma$ H2A.X. \*\*\* $p < 0.001$ .