MC-LR induced overproduction of progesterone via inhibiting miR-3473g: *in vitro* and *in vivo* evidence

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Abstract

Health risk of human exposure to microcystin-leucine arginine (MC-LR) has drawn more and more attention in recent years. In the present study, MC-LR inhibited miR-3473g expression of mouse granulosa cells both *in vitro* and *in vivo*. By dual-luciferase reporter assay, we confirmed miR-3473g is able to bind the 3'-UTR of StAR protein (StAR) mRNA and suppress StAR expression. Thus, downregulation of miR-3473g after MC-LR exposure led to StAR overexpression. Excessive StAR probably transported much more cholesterol into the inner membrane of the mitochondria and finally resulted in overproduction of progesterone. Our results revealed that MC-LR exposure was associated with premature luteinization of granulosa cells and may adversely affect women's fertility.

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Introduction

Due to global warming and over-supply of nutrients (nitrogen and phosphorus) discharge from agricultural waters and wastewaters, the incidences and intensities of cyanobacterial blooms have increased substantially in the past decades (Ballot *et al.* 2005, Michalak *et al.* 2013, Paerl *et al.* 2014, Buratti *et al.* 2017). Cyanobacteria can produce a wide variety of potent toxins and pose a hazard to farming, fishing and human health (Svircev *et al.* 2017, Laszakovits & MacKay 2019). Among these cyanotoxins, microcystin-leucine arginine (MC-LR) attracted most attention owing to its broadest distribution and strongest toxicity (Codd *et al.* 2005, Pearson *et al.* 2010). MC-LR is highly water stable and conventional water treatment technologies cannot effectively remove the extracellular MC-LR (Zhang *et al.* 2012, Jasim & Saththasivam 2017, Jian *et al.* 2019). Thus, MC-LR is prone to pass into human body and impose a serious threat to humans directly through drinking water or indirectly by consumption of MC-LR contaminated aquatic organisms (Chen *et al.* 2018, Palagama *et al.* 2019).

It has been well documented that MC-LR is able to induce hepatotoxicity and neurotoxicity, kidney impairment, and gastrointestinal disorders (Wang *et al.* 2010, Li *et al.* 2015, 2019). In our previous study (Wu *et al.* 2015), environmental level of MC-LR was orally administered to female mice and led to decreased development of follicles, elevated serum progesterone,

irregular estrous cycles and subfertility. As the basic functional unit of the ovary, each follicle is comprised of an oocyte surrounded by somatic granulosa cells. Granulosa cells are responsible for secretion of steroid hormones that are necessary to prepare the reproductive tract for fertilization and the establishment of pregnancy (Knight & Glister 2006, Fan *et al.* 2009). Primary cultured mouse granulosa cells (mGCs) could uptake MC-LR and become the target of the toxicant (Wu *et al.* 2015).

miRNAs are endogenous noncoding RNA molecules approximately 22–25 nucleotides in length. By binding to the complementary sequences on the 3'-UTRs of target mRNAs resulting in mRNA translational inhibition or degradation, miRNAs play a central part in diverse cellular processes, including cell differentiation, proliferation and survival (Bartel 2004, Stark *et al.* 2008). Unsurprisingly, it has been reported that miRNAs play a crucial role in the female reproductive system (Carletti & Christenson 2009, Gay *et al.* 2018). In order to figure out whether miRNAs participated in the toxicity of MC-LR on mGCs, we performed miRNAs microarray technology to detect the expression of miRNAs of mGCs after MC-LR exposure. The results showed that 125 miRNAs changed significantly, including 50 miRNAs downregulated and 75 miRNAs upregulated (Li *et al.* 2017). Among these miRNAs, the downregulated miR-3473g (fold change >2) attracted our attention. StAR protein (StAR), the crucial rate-limiting catalyzing enzymes in progesterone synthesis, was predicted to be the target gene of miR-

3473g by miRanda (<http://www.microrna.org/microrna/home.do>) and TargetScan (<http://www.targetscan.org/>). Downregulation of miR-3473g probably removed its inhibition on StAR and led to increased progesterone synthesis which played a key part in MC-LR-induced female reproductive toxicity. To obtain a complete understanding of abnormal progesterone synthesis after MC-LR exposure, a combined *in vitro* and *in vivo* approach was applied in the present study that focused on miR-3473g.

Materials and methods

Ethics statement

The animal experiments were performed according to the Guide for the Care and Use of Laboratory Animals (The Ministry of Science and Technology of China, 2006) and all experimental protocols were approved under the animal protocol number SYXK (Su) 2014-0052 issued by Jiangsu Provincial Science and Technology Department.

Chemicals and reagents

Microcystin-leucine arginine (purity $\geq 95\%$) was purchased from Alexis Biochemicals (Lausen, Switzerland). One milligram MC-LR was dissolved in 100 μL DMSO and diluted to 1 ml with sterilized water as a stock solution kept at -20°C within six months. Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium-Ham's F-12 (DMEM-F12) medium were purchased from Gibco. Lipofectamine 2000, SYBR GreenER qRT-PCR kits, Taqman microRNA assay and universal PCR Mastermix were from Invitrogen. Progesterone and estradiol ELISA kits were purchased from Enzo Life Sciences (Lausen, Switzerland). Rabbit anti-StAR and mouse anti-GAPDH were obtained from Abcam. Rabbit anti-luteinizing hormone/chorionic gonadotropin receptor (anti-LHCGR) was obtained from Proteintech (Wuhan, China). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit/mouse IgG, which was used as the secondary antibody, was purchased from Boster (Wuhan, China). Enhanced chemiluminescence detection kit was from Alpha (San Jose, CA, USA). Dual-Luciferase Reporter Assay System was purchased from Promega.

Mouse primary granulosa cell culture and treatment

Mouse granulosa cells were collected from the ovaries of 21-day-old immature female BALB/c mice using the follicle puncture method, as described by Kipp *et al.* (2007). Oocytes were filtered out with a 40- μm cell strainer. The mGCs were cultured in DMEM-F12 supplemented with 10% FBS and incubated at 37°C with 5% CO_2 in a humidified chamber. Two days later, mGCs were adherent to the bottom of the dishes and formed a monolayer after another 2 days. The purity of mGCs obtained was above 95% when examined microscopically and the survival percentage of mGCs was above 98% assessed by *in situ* trypan blue staining. MC-LR (stock solution) was dissolved in DMEM-F12 and applied to mGCs at concentrations of 0, 1.25, 5 and 20 μM . 48 h later,

the RNA, protein and cell culture supernatants were harvested to detect the relative expression and hormone secretion of the cells.

For preparing miR-3473g vector transfected mGCs, transfections of miR-3473g mimic, inhibitor, control-mimic, or control-inhibitor was performed by Lipofectamine 2000. After transfection, the plate was placed in a CO_2 incubator at 37°C for 6 h, then the medium of mimic, inhibitor or negative control and lipo2000 mixture was removed, and fresh DMEM-F12 medium containing 10% FBS was replaced for culture. After 24 h of transfection, mGCs were next incubated either in the absence or presence of 5 μM MC-LR for 48 h to harvest the RNA, protein and cell culture supernatant to detect the relative expression and hormone secretion by the cells.

Animals and treatment

Six-week-old female BALB/c mice (specific pathogen-free, SPF) ($n=100$) (18–22 g) were purchased Medical Center of Yangzhou university (Yangzhou, China). Mice were individually housed in laminar flow cabinets under a specific pathogen-free environment with access to food and water *ad libitum*. The animals were acclimatized for 1 week before use, and maintained throughout at standard conditions: 50% relative humidity, 24°C , and 12:12 h light-dark circle. Mice were randomized into ten groups ($n=10$ for each) (Supplementary Table 1, see section on [supplementary materials](#) given at the end of this article). Four groups of mice were intraperitoneally injected with MC-LR at 0, 7.5, 22.5 or 45 $\mu\text{g}/\text{kg}$ body weight every day for 2 weeks. The MC-LR (stock solution) was dissolved in 400 μL saline to achieve required concentrations.

Another six groups received miRNA injection according to the method of Yin *et al.* (2014). Mice were anesthetized using pentobarbital. A small dorsal abdominal incision was made in the lumbar region on each side of the midline. Through the incision in the dorsal abdominal wall, the ovaries and associated fat pad were gently pulled outside. The intrabursal injection was performed under microscopic magnification by inserting a 30-gauge needle with 20 μL physiological saline or 2×10^6 transduction units of lentivirus through the ovarian fat pad into the ovarian bursa of each side. The wound was sutured with silk when the ovaries were returned to the abdominal cavity. These six groups of mice were injected with physiological saline, physiological saline, lentiviral vectors containing mimic-NC, miR-3473g-mimic, inhibitor-NC or miR-3473g-inhibitor (Genechem, Shanghai, China), respectively. One week later, group 5 received intraperitoneally 400 μL saline solution every day for 2 weeks and the remaining groups 6–10 received intraperitoneally 22.5 $\mu\text{g}/\text{kg}$ body weight MC-LR every day for 2 weeks.

Quantitative RT-PCR

Total RNA from mGCs and ovaries was isolated using TRIzol reagent (Invitrogen). The purity of RNA was determined with a spectrophotometer (Hoefer, Holliston, MA, USA). One microgram of total RNA was used for cDNA synthesis. RT was performed using the Superscript III first-strand synthesis system (Invitrogen) or the TaqMan microRNA reverse transcription kit

(Invitrogen) on a Veriti 96-Well Fast Thermal Cycler (Applied Biosystems). Quantitative PCR was performed using the SYBR GreenER qRT-PCR kit or the Taqman microRNA assay and universal PCR Mastermix using a 7900 HT Fast Real-Time PCR System (Applied Biosystems). All the procedures were repeated for three times. For each replication of ovarian tissue, total RNA sample was extracted from one ovary of different mouse in each group. The relative quantification values for each miRNA and mRNA were calculated by the $2^{-\Delta\Delta C_t}$ method using U6 and GAPDH as an internal reference, respectively. The sequences of mRNA primer pairs used in this assay included StAR, forward primer AACGGGGACGAAGTGCTAAG, reverse primer CCGTGTCTTTTCCAATCCTCTG; GAPDH, forward primer AGGTCCGGTGTGAACGGATTG, reverse primer TGTAGACCATGTAGTTGAGGTCA.

Western blotting

For *in vivo* part, one ovary was homogenized for extracting proteins in 1 mL ice-cold RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP, 0.1% SDS, 1× phosphatase/protease inhibitors). For mGCs, 200 µL protein extraction buffer was added into the dish to extract the total protein from cells. The protein concentration was determined using the BCA protein assay kit (Beyotime, Nantong, China). About 20 µg of protein from each sample was separated on 10% SDS-PAGE and electrophoretically transferred to a PVDF membrane. The membrane was then blocked in TBS buffer containing 5% BSA (20 mM Tris-HCl (pH 7.6) and 150 mM NaCl) for 1.5 h at 37°C. Next, these blots were incubated sequentially with primary rabbit anti-StAR, mouse anti-GAPDH and HRP-conjugated secondary antibodies. The chromogenic signal intensity was detected using an Odyssey Scanning System (LI-COR, Lincoln, NE, USA) and quantified using ImageJ software (NIH, Bethesda, MD, USA). All the procedures were repeated for three times. For each replication of ovarian tissue, protein sample was extracted from one ovary of different mouse in each group.

ELISA assay

Progesterone and estradiol levels in cell supernatant or serum were measured by rodent ELISA kits according to the manufacturer's instructions. The detection limits were 0.1 ng/mL for progesterone and 5 pg/mL for estradiol. The serum samples were prepared as described previously (Wu *et al.* 2015). Briefly, blood was collected by removing the eyeballs and placed in 1.5 mL microcentrifuge tubes at 4°C overnight. On the second day, the tubes were centrifuged at 200 g for 30 min at 4°C and the sera were collected.

Immunofluorescence

Collected mGCs were centrifuged at 160 g for 5 min at 4°C and incubated sequentially with 4% paraformaldehyde fixative for 10 min and 3% BSA-blocking agents for 30 min. Primary monoclonal anti-StAR (1:200) was then applied and incubated overnight. Alexa Fluor® 488-conjugated goat anti-rabbit IgG (1:200) and phalloidin (1:1000) was added and samples were

incubated for 1 h at 37°C. Nuclei were counterstained with 5 µg/mL DAPI. Finally, the slides were mounted with glycerin and observed using a confocal fluorescence microscope (Olympus).

Histological evaluation and immunohistochemistry

Ovaries were fixed in 4% paraformaldehyde solution for 24 h, embedded in paraffin and cut into 5-µm-thick sections. For histological evaluation, the ovarian sections were stained with hematoxylin and eosin and observed under a light microscope DXM12000F (Nikon). For immunohistochemistry, the sections were incubated with PBS containing 3% H₂O₂ for 10 min to inhibit endogenous peroxidase activity. After being blocked with 3% BSA in PBS for 1 h at 37°C, the sections were subsequently incubated with rabbit anti-LHCGR (1:200) overnight at 4°C and then with HRP-conjugated secondary antibodies (1:200) at 37°C for 1 h. HRP activity was examined using the diaminobenzidine method according to the manufacturer's instructions (Zhongshan Biotechnology, Beijing, China). After counterstaining with hematoxylin, the sections were mounted with neutral balsam for observation under a microscope DXM12000F.

Dual-luciferase reporter assay

The 3'-UTRs of mouse StAR (Supplementary Table 2) encompassing the conserved miR-3473g response elements were cloned into the GV272 vector (Shanghai Genechem Co., Ltd, China, Supplementary Fig. 1). The 293T cells were seeded in a 24-well plate for 24 h, and transfection was performed when cell density in the plate reached 60%. 100 ng of each GV272-Control or GV272-StAR plasmids were used as reporter constructs and were cotransfected into 293T cells with 25 µmol miRNA mimic (control mimic or miR-3473g-mimic) using Lipofectamine 2000 (Invitrogen). The plate was placed in an incubator for 6 h, and then the medium was removed and fresh complete medium was added. After 24 h, firefly luciferase activity and Renilla activity were determined in cell lysates by Promega Dual Luciferase Assay Kit (Promega (Beijing) Biotech Co., Ltd).

Statistical analysis

SPSS 18.0 (SPSS) was used for statistical analysis. Experimental results were expressed as mean ± s.e. The Levene's test was employed to check the normality and homogeneity of variances in the data. The Student's *t* test was used for paired comparisons. For the comparison of three or more groups, one-way ANOVA was used for the comparison, which was followed by Duncan's *post hoc* test. Values of *P* < 0.05 were considered statistically significant.

Results

MC-LR-induced downregulation of miR-3473g in mGCs and miR-3473g targets StAR mRNA

We treated mGCs with 5 µM MC-LR for 48 h. Then the expression of miR-3473g in mGCs was determined by

qRT-PCR and dual-luciferase reporter assay was performed to verify the target gene of miR-3473g. The results showed that miR-3473g was significantly downregulated (Fig. 1A). Next, we tried to predict the potential targets of miR-3473g by TargetScan and miRanda. StAR stood out as one of the target mRNAs of miR-3473g. The binding sites for miR-3473g in the 3'-UTR of StAR were shown in Fig. 1B. Then we tried to confirm the binding of miR-3473g to the 3'-UTR of StAR in 293T cells using the dual luciferase reporter gene assay system. StAR 3'-UTR and StAR 3'-UTR with mutations in the predicted miR-3473g binding site were cloned separately into the luciferase reporter system (GV272 vector). The GV272 vectors were then co-transfected with either miR-3473g mimic or its negative control. Luciferase activity of the system containing wild-type, but not mutated, StAR 3'-UTR was substantially decreased after co-transfection with miR-3473g mimic (Fig. 1C), which supported the prediction that StAR was the target gene of miR-3473g.

MC-LR exposure led to increased StAR expression and progesterone production of mGCs

Mouse granulosa cells were exposed to 0, 1.25, 5 or 20 μ M MC-LR for 48 h. Quantitative RT-PCR and Western blotting were performed to detect mRNA and protein expression of StAR in mGCs. Meanwhile, progesterone secreted by mGCs in cell culture supernatants were detected by ELISA assay. The data showed that StAR

Figure 1 Effects of MC-LR on miR-3473g in mouse granulosa cells (mGCs) and validation of miR-3473g target gene by luciferase reporter assay. (A) miR-3473g expression in mGCs after 48-h MC-LR exposure at 5 μ M was measured by qRT-PCR. (B) Nucleotide sequences of murine miR-3473g and the 3'-UTR of StAR region, which encompasses the miR-3473g seed match region (bold). (C) Luciferase reporter assays were carried out in 293T cells following co-transfection of miR-3473g mimic negative control (miR-NC) or miR-3473g mimic (miR-3473g) together with empty vector (negative control), mutant (MUT) StAR vector or wild-type (WT) StAR vector, as indicated. Firefly luciferase activity was normalized based on the Renilla luciferase activity. Data are shown as mean $\pm$ S.E. ($n=3$). ** $P<0.01$.

Figure 2 Effects of MC-LR on StAR expression and progesterone production of mouse granulosa cells (mGCs). Granulosa cells were exposed to 0, 1.25, 5 or 20 μ M MC-LR for 48 h. (A) The mRNA levels of StAR in mGCs were determined by qRT-PCR. (B and C) The protein levels of StAR in mGCs were determined by Western blotting (B) and densitometry data are shown (C). (D) The concentrations of progesterone in mGCs cell culture supernatants were determined by ELISA assay after 48-h MC-LR exposure. Data are shown as mean $\pm$ S.E. ($n=3$). * $P<0.05$, ** $P<0.01$.

mRNA and protein in all the MC-LR-treated groups were significantly enhanced (Fig. 2A, B and C). As StAR is the key rate-limiting enzyme in progesterone synthesis, we can reasonably expect the change of progesterone concentration in mGCs cell culture supernatants. The average progesterone concentration in control group was 1855 ± 151 pmol/L, while values in experimental groups treated with 1.25, 5 and 20 μ M MC-LR were 1410 ± 175 , 3344 ± 192 and 2896 ± 298 pmol/L. The coefficients of variation were 14.1, 21.5, 9.9 and 17.8%, respectively. Compared with the control group, progesterone production significantly increased in 5 and 20 μ M MC-LR-treated groups (Fig. 2D).

Over-expression of miR-3473g relieved MC-LR-induced upregulation of StAR expression and progesterone production in vitro

To examine the regulatory effects of miR-3473g on progesterone synthesis of mGCs, we transfected mGCs with miR-3473g-mimic, miR-3473g-inhibitor, and their negative controls followed by exposure to 5 μ M MC-LR for 48 h. The transfection had no significantly effect on cell viability of mGCs (Supplementary Fig. 2). Quantitative RT-PCR was performed to detect miR-3473g and StAR mRNA in mGCs. Western blotting and immunofluorescence were used to detect StAR protein in mGCs. Progesterone secreted by mGCs were detected in cell culture supernatants by ELISA assay. Figure 3A showed that miR-3473g was highly increased in mGCs transfected with miR-3473g mimic and decreased

in mGCs transfected with miR-3473g inhibitor. The expression of StAR mRNA of MC-LR-treated mGCs was suppressed by miR-3473g-mimic and remained high in miR-3473g-inhibitor group (Fig. 3B). The expression of StAR protein showed the same trend as mRNA (Fig. 3C, D and E). At the same time, miR-3473g mimic could reverse MC-LR-induced progesterone production of mGCs (Fig. 3F). Elevated progesterone often comes from premature luteinization of granulosa cells accompanied by a increase in ratio of progesterone to estradiol (P:E ratio). Not surprisingly, P:E ratio in MC-LR group was much higher compared with control group which indicated that mGCs exhibited signs of premature

Figure 3 The effects of miR-3473g on mouse granulosa cells (mGCs). Granulosa cells were transfected with miR-3473g-mimic-negative control (mi-NC), miR-3473g-mimic (mi-3473g), miR-3473g inhibitor-negative control (in-NC), or miR-3473g-inhibitor (in-3473g). Twenty-four hours later, mGCs were incubated with 5 μ M MC-LR for 48 h. (A) Expression of miR-3473g in mGCs was detected by qRT-PCR, (B) The StAR mRNA in mGCs was measured by qRT-PCR. (C and D) The StAR protein in mGCs was detected by Western blotting (C) and densitometry data are shown (D). (E) The StAR protein in mGCs was detected by immunofluorescence. (F) The progesterone in cell supernatant was measured by ELISA assay. (G) The relative ratio of progesterone to estradiol in cell supernatant was measured by ELISA assay. Data are shown as mean $\pm$ S.E. ($n=3$). * $P<0.05$, ** $P<0.01$.

Figure 4 The effects of MC-LR on expression of miR-3473g, StAR in mouse ovaries and serum progesterone. Mice were intraperitoneally injected with MC-LR at 0, 7.5, 22.5 or 45 μ g/kg body weight every day for 2 weeks. (A) The expression of miR-3473g in ovarian tissues was measured by qRT-PCR. (B) The StAR mRNA in ovarian tissues were measured by qRT-PCR. (C and D) The StAR protein in ovarian tissues were measured by Western blotting (C) and densitometry data are shown (D). (E) The concentrations of serum progesterone were determined by ELISA assay. Data are shown as mean $\pm$ S.E. ($n=3$). * $P<0.05$, ** $P<0.01$.

luteinization. Transfection of miR-3473g mimic prevented the increase of P:E ratio (Fig. 3G).

MC-LR inhibited miR-3473g and led to increased StAR and progesterone in vivo

Four groups of mice ($n=10$ for each) were intraperitoneally injected with MC-LR at 0, 7.5, 22.5 or 45 μ g/kg body weight every day. Two weeks later, the expression of miR-3473g was determined in ovarian tissues. miR-3473g was significantly downregulated in a dose-dependent manner (Fig. 4A). We next analyzed StAR expression at the mRNA and protein levels (Fig. 4B, C and D) and the results showed a significant upregulation. However, maximum increment was not observed at the high dose MC-LR treatment group. The high dose of MC-LR may cause other damages besides inhibition of miR-3473g. The average serum progesterone concentrations of 0, 7.5, 22.5 or 45 μ g/kg MC-LR-treated mice were 11.69 ± 0.98 , 23.58 ± 0.33 , 46.85 ± 3.95 and 37.28 ± 1.32 pmol/L. The coefficients of variation were 14.5, 2.5, 14.6 and 6.1%, respectively (Fig. 4E). These results were consistent with our *in vitro* findings.

Over-expression of miR-3473g held back MC-LR-induced upregulation of StAR and progesterone in vivo

Six groups of mice ($n=10$ for each) received intraperitoneal injection of physiological saline, physiological saline, lentiviral vectors containing mimic-NC, miR-3473g-mimic, inhibitor-NC or miR-3473g-inhibitor, respectively. One week later, the control group received intraperitoneally 400 μ L saline solution every day for 2 weeks and the remaining five groups received intraperitoneally 22.5 μ g/kg body weight MC-LR every day for 2 weeks. We found that the mimic increased the expression of miR-3473g, whereas the inhibitor suppressed the expression of miR-3473g (Fig. 5A). We further investigated the effect of manipulated miR-3473g expression on the ovarian StAR and serum progesterone with or without MC-LR exposure. Over-expression of miR-3473g could inhibit the upregulation of StAR induced by MC-LR at both mRNA and protein levels (Fig. 5B, C and D). As the consequence, serum progesterone also declined (Fig. 5E).

MC-LR induced upregulation of LHCGR and premature luteinization of mGCs in ovary which could be reversed by miR-3473g

The remaining ovaries of six groups of mice in Fig. 5 were taken for LHCGR detection and histological evaluation. Compared with control, LHCGR expression of the follicular mGCs was significantly upregulated in MC-LR-treated group. Meanwhile, the upregulation of LHCGR induced by MC-LR was inhibited by miR-3473g (Fig. 6A). As Fig. 6B showed, in the control or combined treatment of MC-LR and mi-3473g groups, the mGCs of antral follicles exhibited normal morphology. By contrast, in the remaining four groups, mGCs of antral follicles presented a clear sign of luteinization. Specifically, the connection between granulosa cells got loose and small round vacuoles appeared. The staining of mGCs nucleus faded and round lipid droplets formed in the cytoplasm of mGCs. These changes demonstrated that MC-LR exposure did trigger the premature luteinization of mGCs and mi-3473g played a pivotal role in this process.

Discussion

MC-LR has triggered increasing concerns about the health risk due to its global distribution. As relatively large hydrophilic molecules, MC-LR enters cells with the assistance of specific membrane transporters named organic anion transporter peptides (OATPs) (Fischer et al. 2005). Following cell entry, MC-LR can cause toxic effects through multiple cellular pathways that may differ according to the duration and approach of exposure. It has been well documented that MC-LR inhibits serine/threonine protein phosphatases (PP) and results in a set

Figure 5 Validating the functions of miR-3473g on ovarian StAR and serum progesterone levels of female mouse. Mice received intraperitoneal injection of physiological saline, physiological saline, lentiviral vectors containing mimic-NC, miR-3473g-mimic, inhibitor-NC or miR-3473g-inhibitor, respectively. One week later, the control group received intraperitoneally 400 μ L saline solution every day for 2 weeks and the remaining five groups received intraperitoneally 22.5 μ g/kg body weight MC-LR every day for 2 weeks. (A) The expression of miR-3473g in ovarian tissues was measured by qRT-PCR. (B) The StAR mRNA in ovarian tissues were measured by qRT-PCR. (C and D) The StAR protein in ovarian tissues were measured by Western blotting (C) and densitometry data are shown (D). (E) The concentrations of serum progesterone were determined by ELISA assay. Data are shown as mean $\pm$ S.E. ($n=3$). * $P<0.05$, ** $P<0.01$.

of negative effects (Zhou et al. 2015). Oxidative stress was often observed due to overproduction of reactive oxygen species or depletion of glutathione in many MC-LR exposure studies (Shi 2009, Puerto et al. 2010, Meng et al. 2015). DNA damage occurred after MC-LR exposure resulting from either oxidative damage (Zegura et al. 2011) or direct interference to DNA damage repair pathways (Zegura et al. 2008). MC-LR has been found to distribute in the liver, kidneys, lungs, brain, heart, gonads and is able to accumulate in organs by low-dose exposures during the whole life (Wang et al. 2008). Dozens of studies focused on male reproductive toxicity of MC-LR. Decrease in testis weight, sperm concentration, level of serum testosterone and damage to spermatogenic epithelium appeared after MC-LR exposure (Li et al. 2008, Wang et al. 2013). There are also some reports about reproductive toxicity of MC-LR on female fish (Kawan et al. 2019, Liu et al. 2019), but the reports of its female mammalian reproductive toxicity

Figure 6 Validating the functions of miR-3473g on luteinizing hormone/chorionic gonadotropin receptor (LHCGR) level and premature luteinization of granulosa cells in mouse ovary. Mice received intraperitoneal injection of physiological saline, physiological saline, lentiviral vectors containing mimic-NC, miR-3473g-mimic, inhibitor-NC or miR-3473g-inhibitor, respectively. One week later, the control group received intraperitoneally 400 μ L saline solution every day for 2 weeks and the remaining five groups received intraperitoneally 22.5 μ g/kg body weight MC-LR every day for 2 weeks. (A) Representative images of LHCGR expression patterns in mouse ovarian tissues determined by immunohistochemistry. The LHCGR-positive cells are in brown (arrow) and the negative cells are in blue. (B) Quantitative analysis of LHCGR expression in ovary using ImageJ software. Data are shown as mean $\pm$ s.e. ($n=3$). * $P<0.05$, ** $P<0.01$. (C) The ovarian histological analysis by hematoxylin-eosin staining shows abnormal premature luteinization of granulosa cells, normal granulosa cells (arrow), luteinized granulosa cells (arrow head), corpus luteum (CL).

are relatively few. Our previous research demonstrated female reproductive toxicity of MC-LR on mice and first discovered that 125 miRNAs of mGCs significantly changed after MC-LR exposure (Wu *et al.* 2015, Li *et al.* 2017). In the present study, we confirmed that miR-3473g in mGCs was downregulated after treatment of MC-LR. By dual-luciferase reporter assay, we established that miR-3473g was able to bind 3'-UTRs of mouse StAR and inhibited StAR expression.

The first step of steroidogenesis occurs within mitochondria. The principal action of StAR is to facilitate the movement of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane (Stocco & Clark 1996, Christenson & Strauss 2000). In the inner mitochondrial membrane, cholesterol becomes substrate of P450scc and is converted to pregnenolone (Simpson & Boyd 1966, Miller 2017). Afterward, pregnenolone is catalyzed by a 42-kDa microsomal enzyme named 3β -HSD and completes the conversion to progesterone (Miller & Auchus 2011).

Following ovulation, luteinized granulosa cells secrete progesterone to establish and maintain early pregnancy (Sellix 2015, Skory *et al.* 2015). However, in the previous follicular phase, a selected cohort of antral follicles grow rapidly. Their steroidogenic activity and response to gonadotrophins are finely modulated to prevent premature luteinization. The inhibition of premature luteinization is required to sensitize certain follicles for further growth and selection of a dominant follicle (Oktem & Uрман 2010). Premature luteinization characterized by elevated progesterone in the late follicular phase has been well reported in the past

30 years. It was supposed to affect 12.3–46.7% of *in vitro* fertilization cycles depending on progesterone (Venetis *et al.* 2013). Endometrial receptivity relies heavily on the duration of progesterone exposure after adequate estrogen exposition. Premature progesterone elevation in patients was demonstrated to affect gene expression profiles in the endometrium and disrupt endometrial receptivity (Labarta *et al.* 2011, Van Vaerenbergh *et al.* 2011). Due to high progesterone, altered epigenetic modification status found in the endometrium may pose a negative impact on endometrial receptivity and lead to lower pregnancy rates (Xiong *et al.* 2017). Furthermore, recent studies reported that elevated progesterone toward the end of the follicular phase had a negative influence on the oocyte or embryo quality (Huang *et al.* 2016, Vanni *et al.* 2017).

To date, the exact cause of progesterone elevation in the late follicular phase is not completely clear (Lawrenz *et al.* 2018). There was a persistent and rapid decline in reproductive functions in recent years. Environmental chemicals have been considered as a potential risk factor contributing to increasing reproductive dysfunction (Messerlian *et al.* 2018, Hipwell *et al.* 2019, Kim *et al.* 2019). Our current study demonstrated that MC-LR exposure induced the elevation of progesterone accompanied by an increase in P:E ratio. We also observed evident upregulation of LHCGR expression and premature luteinization of mGCs in ovaries of MC-LR-treated mice. Downregulation of miR-3473g was a key point for the MC-LR-induced progesterone elevation and premature luteinization of mGCs. Our findings revealed that MC-LR was able to cause progesterone elevation

associated with premature luteinization and threaten female reproductive capacity.

In conclusion, our results indicated that MC-LR could inhibit miR-3473g of granulosa cells both *in vitro* and *in vivo*. As the target of miR-3473g, StAR was significantly upregulated after MC-LR exposure. Excessive StAR probably transported much more cholesterol into the inner membrane of the mitochondria and finally led to overproduction of progesterone. Therefore, we demonstrated that MC-LR exposure is correlated with premature luteinization of granulosa cells and poses a serious threat on reproductive health of women.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/REP-19-0313>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

Conceptualization: J W and X H; Funding acquisition: J W and X H; Project administration: X L, J Z and J T; Writing – original draft: X L, J Z and J W; Writing – review and editing: J W, D L and X H.

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