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Brief Communication

Effects of Griseofulvin on In Vitro Porcine Oocyte Maturation and Embryo Development

Yi-Liang Miao,[1] Xia Zhang,[2] Jian-Guo Zhao,[2,3] Lee Spate,[2] Ming-Tao Zhao,[2] Clifton N. Murphy,[2] Randall S. Prather,[2] Qing-Yuan Sun,[3] and Heide Schatten[1]*

[1]*Department of Veterinary Pathobiology, University of Missouri, Columbia, Missouri*
[2]*Division of Animal Science, University of Missouri, Columbia, Missouri*
[3]*State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, China*

Griseofulvin is an orally administered antifungal drug that affects microtubule formation in vitro and interferes with microtubule dynamics in vivo as clearly shown for mitotic cells in several cell systems. This article reports the effects of griseofulvin on in vitro maturation of porcine oocytes and subsequent effects on embryo development. Our results revealed a concentration-dependent effect on meiotic spindles with 20–40 μM griseofulvin affecting oocyte maturation, and 40 μM affecting fertilization and embryo development. These concentrations of griseofulvin did not affect mitochondrial and cortical granule distribution that also depend on microtubule and cytoskeletal functions during oocyte maturation. Specific effects on the meiotic spindle included spindle disorganization and aberrant chromosome separation displayed as prominent chromosome clusters in oocytes treated with 40 μM griseofulvin. These results strongly suggested that griseofulvin affected porcine oocyte in vitro maturation and following embryo development by disturbing microtubule dynamics. Environ. Mol. Mutagen. 53:561–566, 2012.

Key words: microtubules; cytoskeleton; aneuploidy; meiosis; spindle; fertilization

INTRODUCTION

Griseofulvin is a potent orally administered antifungal drug that affects microtubule formation and disrupts microtubule dynamics in vivo and in vitro [Schatten, 1977; Schatten et al., 1982; Wehland et al., 1977]. Its disruptive effects on mitotic cells have been reported in different cell systems [Grisham et al., 1973; Schatten 1977; Schatten et al., 1982] and aneuploidies caused by griseofulvin have been shown in mitotic cells [Pacchierotti et al., 2002]. In tumor cells, griseofulvin induces multipolar mitoses [Ho et al., 2001; Panda et al., 2005; Rebacz et al., 2007; Schatten 1977] and recent studies have also shown that griseofulvin has an effect on centrosomes in mitotic cells by inhibiting centrosome clustering [Rebacz et al., 2007]. Several studies have also shown varying sensitivities of female mouse germ cells to griseofulvin and reported specific effects on meiotic spindles [Mailhes et al., 1993; Marchetti and Mailhes, 1994, 1995; Marchetti et al., 1992, 1996; Tiveron et al., 1992] and subsequent studies showed aneuploidy and meiotic delay in male mouse germ cells [Shi et al., 1999]. While these studies had focused on chromosomes, our studies are focused on the microtubule cytoskeleton that is critical for all stages of oocyte maturation, fertilization, and subsequent embryo development.

Previous studies had employed the mouse system that displays different drug sensitivities and mechanisms of meiotic spindle formation and fertilization compared with nonrodent mammalian systems. Briefly, the mouse meiotic spindle is formed by aggregation of cytoplasmic centrosomal foci that form cytoplasmic asters and migrate to the spindle poles to form the meiotic spindle; in contrast, no cytoplasmic centrosomes and asters exist in nonrodent oocytes and the meiotic spindle is formed by centrosomal proteins that are formed around a centrosome core structure that allows growth of the meiotic spindle. Further-

Additional Supporting Information may be found in the online version of this article.

*Correspondence to: Heide Schatten, University of Missouri, Department of Veterinary Pathobiology, 1600 E Rollins Street, Columbia, MO 65211, USA. E-mail: SchattenH@missouri.edu

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more, the molecular composition of the mouse meiotic spindle is different in that centrosomes are mainly composed of the centrosomal protein gamma-tubulin while nonrodent meiotic spindle centrosomes are mainly composed of the nuclear mitotic apparatus protein NuMA [Schatten and Sun, 2011a,b]. The different organization, dynamics, and centrosomal composition may be among the reasons for the different drug sensitivities in these different systems. One other significant and important difference between the mouse (rodent) system and nonrodent mammalian systems is the mechanism of fertilization. In the mouse, the sperm's centriole complex becomes destroyed in the oocyte while the cytoplasmic asters reorganize to form the mitotic spindle for first and subsequent embryonic cell divisions. In contrast, in nonrodent mammalian systems, including pig and human, the centriole complex is essential for fertilization and forms the sperm aster, zygote aster, and the mitotic apparatus for all subsequent cell divisions [Schatten et al., 2012; Schatten and Sun, 2009a,b, 2010, 2011a,b]. The present study employs the porcine mammalian system that has been shown in many aspects to be very similar to human germ cells and to be an ideal animal model for studying human diseases [Whyte and Prather, 2011] (Supporting Information Table S1).

Microtubules and centrosomes are critical cellular components for oocyte maturation and they are particularly important for the formation of the centrally located spindle that forms after germinal vesicle breakdown (GVBD) and for subsequent formation of the peripherally located metaphase I (MI) and metaphase II (MII) spindles. Spindle formation is an essential part of oocyte maturation; MI spindle integrity is important for the accurate separation of homologous chromosomes while the MII spindle is critical for the separation of sister chromatids to form a fertilization-competent haploid oocyte. Any dysfunction in the formation of the centrally located spindle and the peripherally located MI or MII spindles may lead to aneuploidy that is one of the major causes for infertility and developmental disorders and it is associated with defective chromosome separation during MI and chromatid separation during MII. The accurate separation of chromosomes in MI and chromatids in MII depends on microtubule and centrosome integrity that may be compromised by griseofulvin and may increase the high percent of aneuploidies that are already well known to be associated with human oocyte maturation.

The microtubule cytoskeleton is important for numerous other functions including mitochondria translocation that plays a significant role in the regulation of the oocyte's metabolism [Krisher and Prather, 2012; Redel et al., 2012] and for cortical granule (CG) migration to the oocyte's cortex during maturation. Mitochondria in oocytes play an important role in providing ATP for fertilization and preimplantation embryo development [Torner et al., 2004]. The distribution of CGs during oocyte maturation has been used as an important criterion to evaluate cytoplasmic maturation as CGs of mature oocytes migrate to the cortex and form a continuous layer under the oolemma to block polyspermic penetration [Damiani et al., 1996]. This article includes an analysis of mitochondrial translocation and cortical granule distribution that both depend on cytoskeletal integrity for accurate function during oocyte maturation.

Because many of the microtubule inhibitors such as nocodazole and colcemid as well as environmental toxic agents such as bisphenol A (BPA) [Schatten and Sun, 2009b] have been shown to affect spindle formation during oocyte maturation, fertilization and development [Schatten and Sun, 2009b], we reasoned that griseofulvin might exert similar effects on microtubule-dependent oocyte maturation, fertilization, and embryo development. Our study used porcine oocytes as representative mammalian system which in recent years has been shown to be a most suitable system for cytoskeletal studies; mechanisms employed in this system for oocyte maturation and fertilization are similar to those known for humans.

MATERIALS AND METHODS

Preparation of Pig Oocytes

Porcine ovaries were obtained from a slaughterhouse and transported to the laboratory while maintained at ~34°C. Follicular fluid from 3–6 mm antral follicles was aspirated with an 18-gauge syringe. Cumulus oocyte complexes (COCs) with uniform cytoplasm and several layers of cumulus cells were selected and rinsed three times in washing medium (TCM 199 medium (Sigma, M2154) supplemented with 0.1% PVA, 3.05 mM D glucose, 0.91 mM sodium pyruvate, 75 mg/mL penicillin, 50 mg/mL streptomycin, 0.57 mM cysteine, and 10 ng/mL EGF). Approximately 50–70 COCs per well were cultured in multidish plates containing TCM 199 medium supplemented with 0.1% PVA, 3.05 mM D glucose, 0.91 mM sodium pyruvate, 10 μg/mL gentamycin, 0.57 mM cysteine, 10 ng/mL EGF, 0.5 μg/mL FSH, and 0.5 μg/mL LH, covered with mineral oil. The oocytes were matured for 42–44 hr at 39°C, 5% CO2 in air.

In Vitro Fertilization

Oocytes treated with or without griseofulvin (Sigma, 856444) were inseminated in a 100 mL drop of modified Tris buffered medium (mTBM, in-house medium) containing 0.2% BSA (W/V) and 2 mM caffeine with frozen thawed spermatozoa (5×10^5 cells /mL). After 4 to 5 hr of insemination, oocytes were removed from the fertilization drop and cultured up to 6 days in 500 μl PZM3 medium containing 4 mg/mL BSA.

Griseofulvin Treatment

Oocytes collected from follicles were randomly incubated in maturation media supplemented with 5, 10, 20, or 40 μM griseofulvin for each separate experiment. Embryos derived from in vitro fertilization were also incubated in PZM3 supplemented with 20 μM griseofulvin.

Evaluation of Spindle and Chromosomes by Confocal Laser Scanning Microscopy

Our studies employed antibodies against α tubulin (1:100; Sigma, F2168) to stain microtubules. The zona pellucida of oocytes was removed with 0.25% pronase. The oocytes were washed in PBS, and then fixed in 4% (W/V) paraformaldehyde in PBS for 1 hr at room tem-

perature. Oocytes were washed three times in PBS, and then placed into 50% methanol for 5 min, 100% methanol for 5 min, and 100% acetone for 5 min to extract lipid droplets that are abundant in pig oocytes. Lipid-extracted oocytes were rehydrated and permeabilized in 1% Triton X 100 (V/V) permeabilization solution (1% Triton X 100, 20 mM Hepes, pH 7.4, 3 mM MgCl2, 50 mM NaCl, 300 mM sucrose, 0.02% NaN3 in PBS) for 2 days. After blocking oocytes with 3% BSA (Sigma, A7030) for 1 hr at room temperature, they were stained with 1:100 anti-α-tubulin-FITC antibody and counterstained with 1 mg/mL DAPI in Vectashield mounting medium (Vector Laboratories, Burlingame, CA) to stain DNA. Finally, oocytes were observed under a laser-scanning confocal fluorescent microscope (Zeiss LSM510 META). Scans were taken through the equatorial plane of the oocyte. Randomly, 10 oocytes from each slide were scanned and recorded with the confocal laser fluorescent microscope. Each treatment was repeated at least three times.

Evaluation of Mitochondrial Distribution by Confocal Laser Scanning Microscopy

A stock solution of MitoTracker Red CMXRos (Molecular Probes, Eugene, OR) fluorescence probe at a concentration of 1 mM was prepared in dimethyl sulfoxide and stored at –20°C. Following IVM culture, ocytes at the MII stage were stained for active mitochondria detection in maturation medium containing 0.5 mmol/L cell permeant MitoTracker Red CMXRos for 30 min at 39°C in a dark environment and 5% CO2 in air. After washing three times with maturation medium for 20 min each, oocytes were fixed with 4% (w/v) paraformaldehyde in PBS for 30 min at room temperature, then washed twice in PBS containing 0.3% (w/v) PVA (Sigma, 341584) and 1% (v/v) Triton X-100 (Sigma, T8787), for 10 min each. Oocytes were counterstained with 1 mg/mL DAPI in Vectashield mounting medium (Vector Laboratories, Burlingame, CA) and observed under a laser-scanning confocal fluorescent microscope (Zeiss LSM510 META).

Evaluation of Cortical Granule Distribution by Confocal Laser Scanning Microscopy

Denuded oocytes were fixed with 4% (w/v) paraformaldehyde in PBS for 30 min at room temperature, and then washed three times in PBS containing 0.3% BSA and 100 mM glycine (Sigma, G8898) for 5 min each. After a 5 min treatment with PBS containing 0.1% Triton X-100, oocytes were washed two additional times in PBS (5 min each). To label the cortical granules, oocytes were cultured in FITC-labeled peanut agglutinin (100 mg/mL; Sigma, L7381) in PBS for 30 min in a dark box. After staining, oocytes were washed three times in PBS containing 0.3% BSA and 0.01% Triton X-100. Oocytes were counterstained with 1 mg/mL DAPI in Vectashield mounting medium (Vector Laboratories, Burlingame, CA) and observed under a laser-scanning confocal fluorescent microscope (Zeiss LSM510 META).

Data Analysis

For each treatment, three replicates were used. Statistical analyses were carried out by analysis of variance. Differences between treated groups were evaluated with the Duncan multiple comparison test. Data are expressed as mean ± SEM and $P < 0.05$ were considered significant. The software used was SPSS (Statistics Package for Social Science).

RESULTS

Effects of Griseofulvin on In Vitro Porcine Oocyte Nuclear Maturation, In Vitro Fertilization, and Embryo Development

Proper microtubule and centrosome dynamics are essential for normal oocyte maturation. Abnormalities in these processes may result in abnormal fertility. When GV porcine oocytes were cultured in maturation medium containing 5 or 10 μM griseofulvin, no significant difference on the percent of oocytes that reached the metaphase I stage (oocyte maturation) was observed between control and griseofulvin-treated oocytes. The percent of oocyte maturation was above 65% in both groups. However, when the concentration of griseofulvin was increased to 20 or 40 μM, the maturation percentage of porcine oocytes was decreased to 21.6% and 5%, respectively (Fig. 1, Supporting Information Table S2).

When these matured oocytes were fertilized in vitro, no significant difference on the fertilization percentage, measured as completion of at least the first mitotic division, was observed between the control and groups treated with 5, 10, and 20 μM griseofulvin. However, when the griseofulvin concentration was increased to 40 μM, the fertilization percentage was significantly decreased to 64.5% (Fig. 2, Supporting Information Table S3). After the fertilized oocytes were cultured in vitro, the blastocyst percentage was significantly decreased as the concentration of griseofulvin was increased. Only one of the

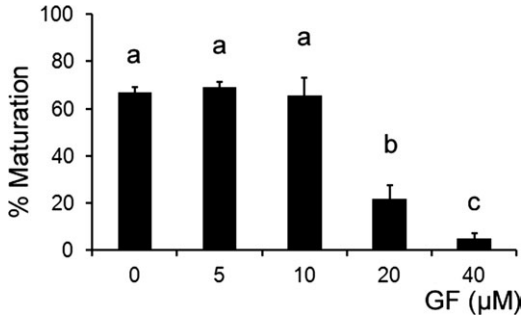

Fig. 1. Effects of griseofulvin on porcine oocytes nuclear maturation in vitro. GV oocytes were treated with 5, 10, 20, and 40 μM griseofulvin during in vitro maturation for 42–44 hr and then evaluated for maturation. Data expressed as mean ± SEM of three experiments. Groups with different letters (a, b, and c) are significantly different, $P < 0.05$, ANOVA.

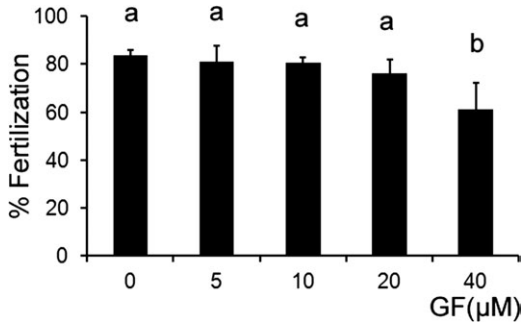

Fig. 2. In vitro fertilization of porcine oocytes treated with griseofulvin. Matured oocytes treated with 5, 10, 20, and 40 μM griseofulvin were fertilizated in vitro and then evaluated for fertilization (cleavage). Data expressed as mean ± SEM of three experiments. Groups with different letters (a, b, and c) are significantly different, $P < 0.05$, ANOVA.

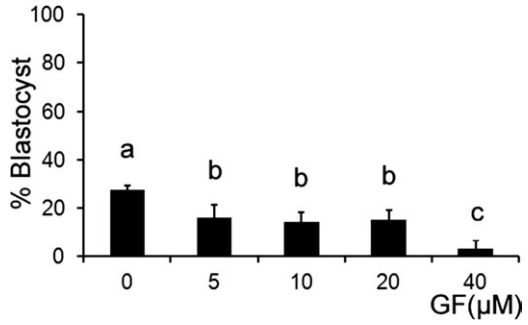

Fig. 3. Development of fertilized porcine oocytes treated with griseofulvin. Fertilized oocytes treated with 5, 10, 20, and 40 μM griseofulvin were cultured in vitro and then evaluated for development to the blastocyst stages. Data expressed as mean ± SEM of three experiments. Groups with different letters (a, b, and c) are significantly different, $P < 0.05$, ANOVA.

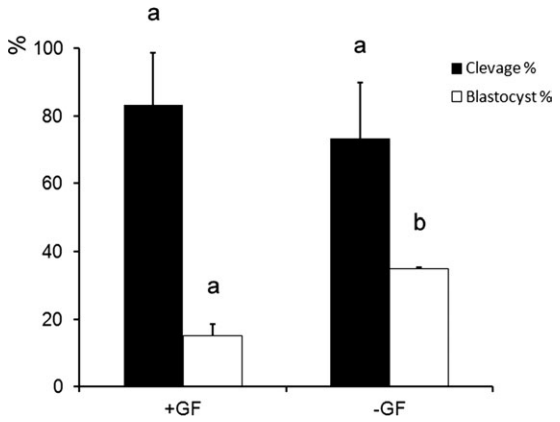

Fig. 4. Effects of griseofulvin on the development of porcine oocytes after fertilization. Normal fertilizated oocytes were culturated in PZM3 with 20 μM griseofulvin and then evaluated for development to the blastocyst stages. Data expressed as mean ± SEM of three experiments. Groups with different letters (a, b, and c) are significantly different, $P < 0.05$, ANOVA.

matured oocytes treated with 40 μM griseofulvin developed to blastocyst stages after fertilization (Fig. 3, Supporting Information Table S3).

Effects of Griseofulvin on Development of Porcine Embryos In Vitro

Based on the results shown above, we next determined whether 20 μM griseofulvin was able to affect postfertilization development of porcine oocytes. Fertilized oocytes without previous griseofulvin treatment were cultured in PZM3 containing 20 μM griseofulvin. The results showed that griseofulvin was able to significantly decrease the blastocyst percentage from 34.8% to 15.2% (Fig. 4).

Effects of Griseofulvin on Spindle Morphology of Porcine Oocytes Matured In Vitro

When the concentration of griseofulvin was increased from 5 μM to 20 μM, the morphology of spindles in matured oocytes still appeared normal. All spindles were at the second meiotic metaphase and displayed the well-known spindle organization with compact chromosomes aligned properly at the equatorial plate (Figs. 5A1–5A4). However, when the concentration of griseofulvin was increased to 40 μM, more than 90% of oocytes displayed abnormal spindle morphology with the chromosomes separating into distinct clusters (Fig. 5A5).

Effects of Griseofulvin on Mitochondrial Distribution of In Vitro Matured Porcine Oocytes

In all examined oocytes, mitochondrial aggregates formed large clusters in the submembranous/pericortical area of the oocyte (Figs. 5B1–5B5) which is typical for this species and indicates proper mitochondrial distribution. There was no difference in mitochondrial distribution between the control and griseofulvin-treated groups, perhaps indicating that the more stable cytoplasmic microtubules important for mitochondrial translocation are not affected by the doses of griseofulvin used in this study in contrast to the more labile microtubules of the meiotic spindle.

Effects of Griseofulvin on Cortical Granule Distribution of In Vitro Matured Porcine Oocytes

In all examined oocytes, nearly all cortical granules migrated to the cortex and became localized just beneath the plasma membrane where they formed a monolayer (Figs. 5C1–5C5) which is typical for this species. There was no difference in cortical granule distribution between the control and griseofulvin-treated groups.

DISCUSSION

In this article we have shown for the first time that griseofulvin affects microtubule dynamics during oocyte maturation in a concentration-dependent manner. We showed normal spindle organization with compact chromosomes aligned properly at the equatorial plate in control cells and in oocytes treated with 5–20 μM griseofulvin; however, when the concentration of griseofulvin was increased to 40 μM, the spindle shape became disorganized and chromosomes were separated into distinct clusters (Figs. 5A1–5A5) and decreases in oocyte maturation, fertilization rate, and first-cleavage metaphase zygotes were observed. These concentration-dependent effects were consistent with in vivo findings about a threshold dose-response relationship for aneuploidy induction during mouse meiosis I and II [Marchetti et al., 1996].

Griseofulvin is a potent antifungal drug that affects microtubules in varied cell systems including tumor cells. Our data shows that its effect on oocyte maturation falls

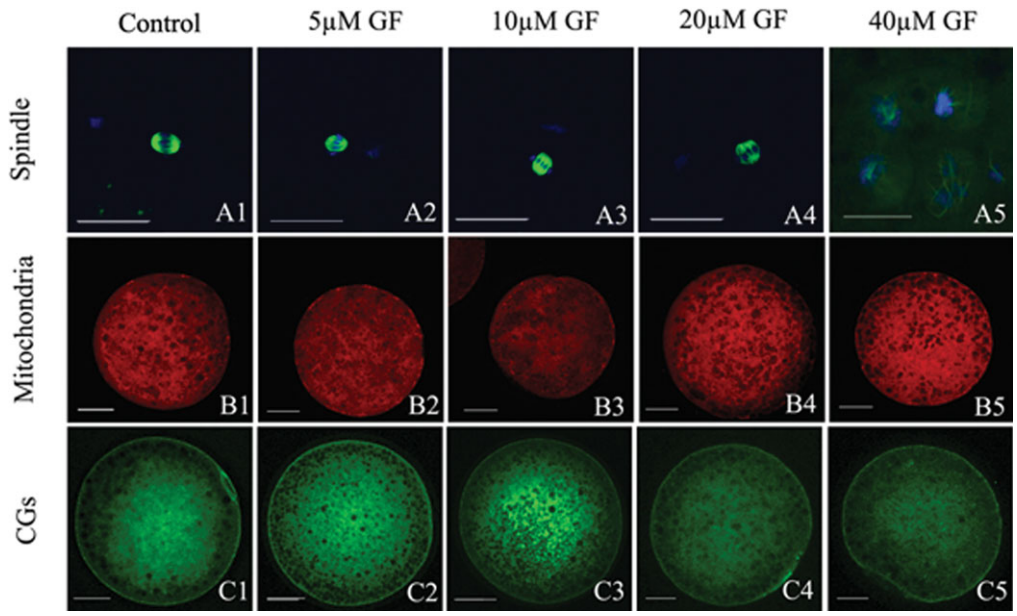

Fig. 5. Confocal micrographs of porcine oocytes treated with 5, 10, 20, and 40 μM griseofulvin showing spindle morphology (green) and chromosomes (blue) (A1-A5), distribution of mitochondria (red) (B1-B5) and distribution of cortical granules (green) (C1-C5). Bar = 40 μM. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

in an important area of studying potential side effects of this orally administered drug. In a previous paper, we have reported the effects of griseofulvin on fertilization in the sea urchin model in which the formation of microtubule-based structures was prevented at 4-6 x 10^{-5} and 1 x 10^{-4} M. Formation of the sperm aster and pronuclear migration as well as the formation of the mitotic apparatus during cell division were affected [Schatten et al., 1982]. These studies also showed that DNA duplication continued in griseofulvin-treated eggs resulting in cell cycle imbalances and formation of multipolar mitoses when eggs were allowed to recover from griseofulvin treatment. We had also studied the effects of griseofulvin on microtubule-based functions in mouse oocytes in which microtubule formation leading to pronuclear apposition were prevented by 100 μM griseofulvin [Schatten et al., 1985].

While studying the effects on the meiotic spindle is clearly most important in regard to aneuploidy, several other events that are important for oocyte maturation and embryonic development are dependent on microtubules including cortical granule migration and mitochondria translocation. However, we found that griseofulvin had no effects on them. It is known that microtubules display different sensitivities in cells; meiotic and mitotic microtubules are more labile than cytoplasmic microtubules that play a role in the cortical granule and mitochondria translocations. In vitro biochemical studies on isolated microtubules [Schatten, 1977] and in vivo studies [Schatten et al., 1982, 1985] had shown different sensitivities to griseofulvin. Overall, these findings indicate there are different drug sensitivities between the labile meiotic spindles and the more stable cytoplasmic microtubules.

Taken together, our research showed that griseofulvin affected oocyte maturation and embryo development in a concentration-dependent manner. High concentration of griseofulvin disrupted spindle formation; however, there were no effects on mitochondrial and cortical granule distribution. These data are relevant for patients who use griseofulvin for fungal infections of the skin and/or the reproductive system. Further studies are needed to measure the effects on the centrosome and microtubule-related processes that are important for oocyte maturation, fertilization, and embryo development [Schatten and Sun, 2011a,b]. These include (a) spindle formation in the center of the oocyte during maturation; (b) spindle migration to the oocyte periphery; (c) MI spindle formation; (d) MII spindle formation; (e) sperm aster formation after fertilization; and (f) formation of the mitotic apparatus during first division.

STATEMENT OF AUTHOR CONTRIBUTIONS

YL Miao and H Schatten designed the study, wrote the paper and applied for Research Ethics Board approval. YL Miao and X Zhang performed the experiments, analyzed the data and prepared draft figures and tables. JG Zhao, MT Zhao, L Spate and C Murphy performed oocyte collections, oocyte maturation, IVF and embryo culture. R Prather and QY Sun revised the manuscript. All authors approved the final manuscript.

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