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In vitro Maturing Mouse Oocytes Treated by Okadaic Acid – Effect on Cytoskeletal Structures and the Chromosome Spread

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Okadaic acid (OA) was applied in different concentrations for 1 or 2 hours on *in vitro* maturing and ovulated mouse oocytes. Actin, tubulin and chromatin were fluorescently stained and chromatin condensation, chromosome spreading, presence and morphology of meiotic spindle and actin cap were analyzed. One hour OA treatment of *in vitro* maturing oocytes produced slender spindles and was not enough for chromosome condensation. *In vitro* maturing oocytes treated for 2 h had abnormal or destroyed spindles and often condensed chromatin. When chromosomes were well spread, actin cap was lacking and actin was distributed uniformly in cortical cytoplasm. These changes preceded changes in meiotic spindle and chromatin. Chromatin condensation was higher for *in vivo* ovulated oocytes and good quality chromosome plates were obtained from them. In conclusion, OA treatment of oocytes affects the actin cap first, causes spindle abnormalities depending on meiotic maturation stage and provides good chromosome spreading and condensation.

Key words: oocyte, actin, tubulin, metaphase plate, okadaic acid.

Introduction

Okadaic acid ($C_{44}H_{68}O_{13}$) is a cytotoxin produced by dinoflagellates. It is named after the marine sponge *Halichondria okadai,* from which this compound was first isolated. Its hydrophobic backbone enables it to enter cells where it stimulates intracellular protein phosphorylation. Okadaic acid (OA) is a reversible, potent and selective inhibitor of serine threonine protein phosphatases PP2A and PP1, especially the former [6]. In oocytes, OA induces activation of MPF, entry to M-phase and chromosome condensation. In *Xenopus* eggs, OA provokes lamin depolymerization and nuclear envelope breakdown and strongly affects the microtubule organizing center [5]. Because of its effect on chromosome condensation, okadaic acid was applied on human second polar bodies to obtain readable metaphase plates as an approach for preimplantation diagnostics in assisted reproduction [7]. The authors reported some benefits of OA treatment (no need of hypotonic incubation during oocyte fixation) but also a disadvantage (cell

fragmentation). According to [1], OA prevents polar body extrusion and causes ruffling of cell membrane and formation of large vacuoles in the ooplasm. Treatment of canine oocytes of different sizes with 0.5 μM OA for 1 h reportedly improves meiotic maturation [2]. These data, however, are still preliminary and no benefit of OA as methodological tool for oocytes is generally accepted. To study the potential of okadaic acid in this respect, we investigated its effects on chromosome condensation and spreading as well as on tubulin and actin cytoskeleton in ovulated and *in vitro* maturing mouse oocytes.

Materials and Methods

The study used 141 mouse oocytes obtained after hormonal stimulation of the animals. Ninety-one oocytes were obtained by ovarian section and subjected to *in vitro* maturation (IVM). Fifty oocytes were isolated from the oviduct after their *in vivo* ovulation. The stimulation was carried out by FSH (Follicle Stimulating Hormone) treatment of animals for the oocytes subjected to *in vitro* maturation and a combination of FSH/hCG (human Chorionic Gonadotropin) for ovulated oocytes.

Three different concentrations (0.4 μM, 2 μM and 5 μM) of okadaic acid were applied for 1 or 2 hours to mouse oocytes at different maturation stages (in vitro maturing or ovulated). Oocytes were then subjected to fluorescent detection of tubulin, actin and chromatin or to classical karyotyping. In the latter case, hypotonic incubation was omitted in some samples. Chromatin was visualized by Giemsa for karyotyping and by HOECHST 33258 (Sigma-Aldrich) for fluorescent analysis. Monoclonal mouse anti-α-tubulin antibody followed by FITC-conjugated goat antibody (Sigma-Aldrich) were used for detection of tubulin. Fibrillar actin was visualized using its specific reaction with phalloidin-TRITC (Sigma-Aldrich).

The results were analyzed regarding chromatin condensation, chromosome spreading, presence and morphology of meiotic spindle and actin cap.

Results

In vitro maturing oocytes

Incubation of in vitro maturing oocytes with OA in different concentrations for 1 hour did not cause chromosome spreading. A slender meiotic spindle containing few fibers was present in these oocytes. The proportion of oocytes with visible spindles was comparable between samples exposed to low and high concentration of OA: 36.36% of oocytes treated by 0.4 μM OA and 32-34% of cells treated by 2 μM or 5 μM OA.

Chromosome spreading was obtained in 26.67% of oocytes incubated for 2 h in 5 μM OA, 30.77% of oocytes incubated in 2 μM OA and 31.25% of oocytes incubated in 0.4 μM OA. The chromosomes were condensed at regard-

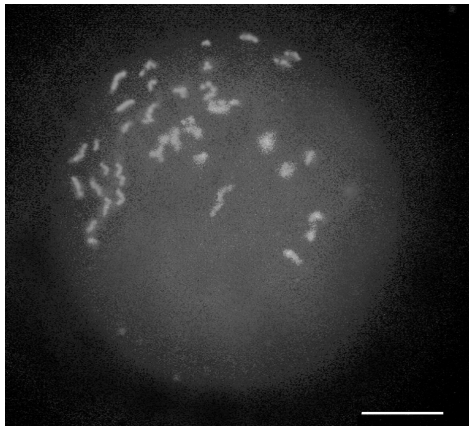

Fig. 1. Spread of chromosomes in an *in vitro* maturing oocyte treated by 5 μM OA for 2 h. The chromosomes are well condensed and distributed in the ooplasm. Most of them are seen represented by single chromatids and their number is corresponding to the diploid set. Bar = 20 μm

less of the stage of meiotic maturation and the spread occupied nearly half of the oocyte volume (**Fig. 1**).

Meiotic spindles had abnormal shape and/or length or were fully destroyed. The low (0.4 μM) or high (2 μM and 5 μM) concentration of OA had different effect on tubulin: spindles were present in 43.75% of oocytes incubated in 0.4 μM OA compared to 23% and 20% respectively of cells treated by 2 μM and 5 μM OA.

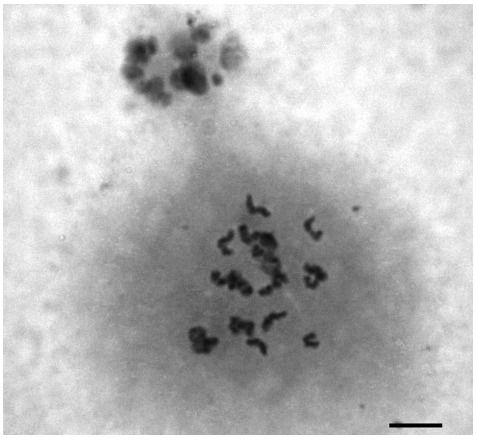

Fig. 2. Good quality chromosome spread of an *in vivo* ovulated oocyte incubated in 2 μM OA for 2 h. Chromosomes have good morphology and condensation and are suitable for classical cytogenetics. Bar = 20 μm

***In vivo* ovulated oocytes**

The incubation in OA for two hours resulted in very good chromosome spreading. Ovulated mouse oocytes had well spread metaphase chromosomes after incubation with OA for 2 hours at concentrations of 2 μM (60%) and 5 μM (75%). No microtubules were observed in these cells. In cases when chromosomes were not spread, the peripheral region adjacent to the chromosomes showed diffuse fluorescence for tubulin.

Good quality chromosome spreads suitable for classical cytogenetic analysis (**Fig. 2**) were obtained from *in vivo* ovulated oocytes incubated in OA for 2 h (58.33% of cells treated by 5 μM OA and 56.25% of those treated by 2 μM OA).

The quality of spreads was slightly variable: most of them had well condensed chromosomes but some had chromosomes longer than normal or not well condensed. On average, spreading was better in cells subjected to hypotonic incubation, therefore this protocol was used for most samples.

Actin cap was lacking in cells with well spread chromosomes and actin was distributed uniformly in the cortical cytoplasm. These changes in actin cytoskeleton preceded changes in meiotic spindle and chromatin.

All groups of oocytes treated by OA had intact cell membrane and no vacuoles or cell fragments were observed.

Discussion

Our study provides data about the sequence of effects caused by OA on actin and tubulin cytoskeleton and their relation to chromosome condensation. Treatment by okadaic acid affects the actin cytoskeleton first and causes abnormalities of meiotic spindle depending on the maturation stage of the oocyte. By our opinion, these dramatic cytoskeletal changes make controversial the stimulating effects of OA on oocyte maturation [2] even if reversibility of the treatment is taken into account.

While okadaic acid suitability to improve the outcome of assisted mammalian reproduction is questionable, it has a potential use in cytogenetic analysis by increasing the yield of good metaphase plates from oocytes. In our study, chromosome condensation was achieved for both mature and immature mouse oocytes after treatment by okadaic acid. The main obstacles in mammalian oocyte cytogenetic analysis are the low number of cells for analysis (especially for human oocytes) and the quality of chromo-

some spreads [8]. Increasing the number of analyzable cells could improve both classical and molecular cytogenetic analysis.

The advantage of OA treatment cited in [7] – skipping of hypotonic incubation, was not confirmed by this study. Our previous experience with mammalian oocyte chromosome spreads [3, 4] has shown strain-dependent differences in chromosome clumping. In the present study, spreading of mouse chromosomes fixed for cytogenetics was better when hypotonic incubation was applied during fixation.

The data reported in [1, 7] concerning changes of oocyte membrane or integrity caused by OA were also not supported by our results. Even after oocyte incubation for 2 hours in the highest (5 μM) concentration of OA, cell membrane was intact and no vacuoles or cell fragments were formed.

In conclusion, our study of the effects of okadaic acid treatment on oocyte chromosomes and cytoskeleton showed that this procedure can be used to obtain metaphase plates suitable for karyotyping from both mature and immature oocytes.

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References

1. **Alexandre, H., A. van Cauwenberge, Y. Tsukitani, J. Mulnard.** Pleiotropic effect of okadaic acid on maturing mouse oocytes. – Development, **112**, 1991, 971-980.
2. **Ariu, F., S. Fois, D. Bebbere, S. Ledda. I. Rosati, M. T. Zedda, S. Pau, L. Bogliolo.** The effect of okadaic acid on meiotic maturation of canine oocytes of different size. – Theriogenology, **77**, 2012, 46-52.
3. **Delimitreva, S., M. Markova, R. Zhivkova.** Optimal metaphase plate preparation in mouse oocytes requires specific conditions for spindle disintegration. – Compt. rend. Acad. bulg. Sci., **52**, 1999, 105-108.
4. **Delimitreva, S., R. Zhivkova, E. Isachenko, N. Umland, P. L. Nayudu.** Meiotic abnormalities in in vitro-matured marmoset monkey (*Callithrix jacchus*) oocytes: development of a non-human primate model to investigate causal factors. – Hum. Reprod., **21**, 2006, 240-247.
5. **Rime, H., D. Huchon, C. Jessus, J. Goris, W. Merlevede, R. Ozon.** Characterization of MPF activation by okadaic acid in *Xenopus* oocyte. – Cell. Differ. Dev., **29**, 1990, 47-58.
6. **Valdiglesias, V., M. V. Prego-Faraldo, E. Pásaro, J. Méndez, B. Laffon.** Okadaic acid: more than a diarrheic toxin. – Mar. Drugs, **11**, 2013, 4328-4349.
7. **Verlinsky, Y., S. Evsikov.** Karyotyping of human oocytes by chromosomal analysis of the second polar bodies. – Mol. Hum. Reprod., **5**, 1999, 89-95.
8. **Zhivkova R. S., S. M. Delimitreva, D. I. Toncheva, I. T. Vatev.** Analysis of human unfertilized oocytes and pronuclear zygotes-correlation between chromosome/chromatin status and patient-related factors. – Eur. J. Obstet. Gynecol. Reprod. Biol., **130**, 1, 2007, 73-83.

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