Alteration of Spindle Formation and Chromosome Alignment in Post-Ovulatory Aging of Mouse Oocytes

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ABSTRACT : The objective of this study was to elucidate the dynamics of microtubules in post-ovulatory aging *in vivo* and *in vitro* of mouse oocytes. The fresh ovulated oocytes were obtained from oviducts of superovulated female ICR mice at 16 hours after hCG injection. The post-ovulatory aged oocytes were collected at 24 and 48 hours after hCG injection from *in vivo* and *in vitro*, respectively. Immunocytochemistry was performed on β-tubulin and acetylated α-tubulin. The microtubules were localized in the spindle assembly, which was barrel-shaped or slightly pointed at its poles and located peripherally in the fresh ovulated oocytes. The frequency of misaligned metaphase chromosomes were significantly increased in post-ovulatory aged oocytes after 48 hours of hCG injection. The spindle length and width of post-ovulatory aged oocytes were significantly different from those of fresh ovulated oocytes, respectively. The staining intensity of acetylated α-tubulin showed stronger in post-ovulatory aged oocytes than that in the fresh ovulated oocytes. In the aged oocytes, the spindles had moved towards the center of the oocytes from their original peripheral position and elongated, compared with the fresh ovulated oocytes. Microtubule organizing centers were formed and observed in the cytoplasm of the aged oocytes. On the contrary, it was not observed in the fresh ovulated oocytes. The alteration of spindle formation and chromosomes alignment substantiates the poor development and the increase of disorders from the post-ovulatory aged oocytes. It might be important to fertilize on time in ovulated oocytes for the developmental competence of embryos with normal karyotypes.

Key words : Cytoskeleton, Microtubule, Acetylated tubulin, Spindle, Chromosome alignment, Post-ovulatory aging, Mouse oocytes

INTRODUCTION

Microtubules and microfilaments are major elements of cytoskeletons in the mammalian oocytes that provide the framework for chromosomal movement and cell division. During meiotic maturation in mammalian oocytes, considerable chromosomal and cytoplasmic changes with cytoskeletons occur including germinal vesicle breakdown (GVBD), chromosomal condensation, polar body extrusion, and formation of the meiotic spindle. These structural changes are associated with reorganization of microtubules and microfilaments during specific phases of the cell cycle. At the time of ovulation in mammals, the ovulated oocytes reach and become arrested at metaphase of the second meiotic division (metaphase II arrest). Fertilization triggers the completion of meiosis and thus entry into the first mitotic cell cycle.

The developmental capacity of the post-ovulatory aged oocytes is much lower than that of fresh ovulated oocytes, which may be based on structural alterations in the spindle, chromosomes, or cytoskeletons during the aging (Marco et al., 1984; 1986). Eichenlaub-Ritter et al. (1986) observed that in aged mouse oocytes the microtubules are gradually lost from the spindle, this results in reduction of pole-to-pole distance in the spindle. The changes of cytoskeletal organization in mouse oocytes have been suggested to be closely related to the different types of parthenotes obtained after activation (Webb et al., 1986). In post-ovulatory aging of mouse oocytes, mitotic arrest deficient (MAD) transcripts

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were decreased and it related to increase frequencies of premature centromere separation and anaphase (Steuerwald et al., 2005). Aged spindles display alteration patterns of centrosome abnormalities and polar structures of spindles in human and porcine oocytes (Miao et al., 2009a; Miao et al., 2009b).

Stabilization and rearrangement of microtubules are necessary for the completion of meiosis, pronuclear apposition, and mitosis from ovulation to first cleavage. A post-translational modification of tubulin acetylation has been found in axonemal microtubules (L'Hernault & Rosenbaum, 1983, 1985; LeDizet & Piperno, 1986), in subsets of cytoplasmic microtubules of some cell types (Piperno & Fuller, 1985; Piperno et al., 1987; Bulinski et al., 1988) and developmental stages (Piperno & Fuller, 1985; Diggins & Dove, 1987; Sasse et al., 1987; Wolf et al., 1988). Some researchers (Piperno et al., 1987; Schulze et al., 1987; Webster & Borisy, 1989) have recently noted a correlation between acetylation and microtubule stability; however, the connection between this post-translational modification and stability is not yet understood. Recently, PADI-6-null oocytes showed very low levels of acetylated microtubules in the ooplasm, and it may relate to organelle redistribution failure (Kan et al., 2011).

Despite the importance of microtubules assembly and alteration patterns in aged mammalian oocytes, little information is available on this subject. In this study, it was examined the dynamics and alterations of microtubules in post-ovulatory *in vivo* or *in vitro* aging of mouse oocytes, and determined the post-translational modification of microtubules to acetylation in the aged oocytes. There may be tentative evidences suggesting that the post-ovulatory aged oocytes should incline to aneuploid embryo.

MATERIALS & METHODS

1. Collection of the Fresh Ovulated and Aged Oocytes

The female ICR mice (10 to 12 weeks old) were superovulated by intraperitoneal injection of 5 IU of pregnant mare's serum gonadotropin (PMSG, Sigma, St. Louis, MO, USA) followed by human chorionic gonadotropin (hCG, Sigma) 48 hours later. The fresh ovulated oocytes were collected by puncturing the ampullae of the oviducts of superovulated female ICR mice at 16 hours after hCG injection. Only the normal matured oocytes showing normal morphology and first polar body, were selected for this experiment. The *in vivo* aged oocytes were obtained from oviducts at 24 hours and 48 hours after hCG injection, respectively. The *in vitro* aged oocytes were prepared at 24 hours and 48 hours after hCG injection during *in vitro* culture of the fresh ovulated oocytes. The HEPES-buffered T6 media with 0.4% bovine serum albumin (Sigma) was used as the collection and *in vitro* culture media. Cumulus cells were removed by brief exposure to the oocyte-cumulus complexes with 0.1% hyaluronidase (Sigma) and repeated pipetting.

2. Immunocytochemistry and Fluorescence Microscopy

All reagents for immunocytochemistry were purchased from Sigma (St. Louis, MO. USA). At specific time points, the oocytes were exposed to a microtubule stabilizing buffer (Simerly & Schatten, 1993; 25% glycerol, 50 mM KCl, 0.5 mM $MgCl_2$, 0.1 mM ethylenediaminetetraacetic acid, 1 mM ethylene glycol tetraacetic acid, 1 mM mercaptoethanol, 50 mM imidazole and 4% Triton X-100, pH 6.7) for 30 minutes. The oocytes were then fixed with 2% neutral buffered formalin for 2 hours. After several washes with phosphate buffered saline (PBS, pH 7.2), the oocytes were incubated in a blocking solution (0.1 M glycine, 0.01% Triton X-100, 1% powder milk, 0.5% BSA and 0.02% sodium azide) for 1 hour.

Immunocytochemistry was performed using mouse monoclonal antibody to Cy3 conjugated β-tubulin and acetylated α-tubulin as primary antibody, and FITC conjugated goat antibody to mouse IgG as secondary antibody. Reaction with the primary antibodies was carried out for 1 hour. After a wash with the blocking solution and several washes with PBS, labeling with the secondary antibody was performed for 1 hour. After blocking and washing, the chromosome was stained with 10 ㎍/㎖ bisbenzimide (Hoechst 33342).

Microscopic observation was performed on a Nikon Optiphot-2 fluorescent microscope (Nikon, Japan) and photographs were taken to determine the pole-to-pole distance and width of spindles. Only the spindles, straight and flatly spread out in one plane of view, were evaluated.

3. Statistical Analysis

The incidence of misaligned chromosomes with spindle in the fresh ovulated and the aged oocytes were compared by Fisher's exact test. The difference of spindle length and width were analyzed by Student's *t*-test. Value of $p<0.05$ was considered statistically significant.

RESULTS

1. Microtubules Organization in the Fresh Ovulated and the Aged Oocytes

In the fresh ovulated metaphase II oocytes, microtubules were observed only in the spindle, which located mainly in periphery position around first polar body (Fig. 1). Microtubule organizing centers (MTOCs) were observed in the pericentriolar region of the spindles and showed ring- or disk-like formations at the poles of the metaphase spindle. Microtubules seemed to fill the region between the poles and the chromosomes in a uniform fashion in the fresh ovulated oocytes. In the aged oocytes, the spindles were had moved towards the center of the oocytes from their original peripheral position and elongated, compared with the fresh ovulated oocytes. Several MTOCs were observed in the cytoplasm of the aged oocytes *in vitro* for 48 hours (Fig. 1).

2. Alterations of Chromosome Alignment and Spindle Formation in the Aged Oocytes

Chromosomes in the fresh ovulated oocytes were located right at the spindle equator in the center of the spindle, and showed highly ordered alignment at the equator of the metaphase II spindle (Fig. 2A). Cytoplasmic MTOCs and misaligned chromosomes were observed in the aged oocytes

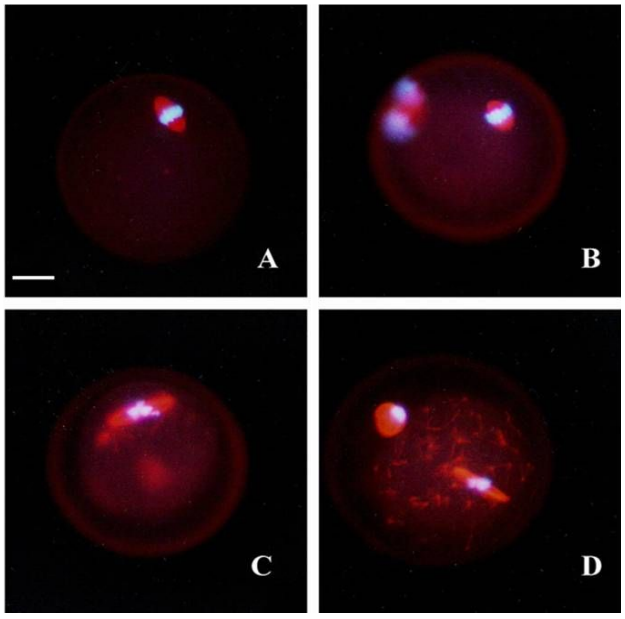

Fig. 1. Fluorescent microphotographs of the microtubules in the mouse fresh ovulated and the aged oocytes. A and B, Microtubules (red color) and chromosomes (blue color) in a fresh ovulated oocyte. C and D, Microtubules and chromosomes in an aged oocytes *in vitro* for 48 hours. Bar indicates 10 ㎛.

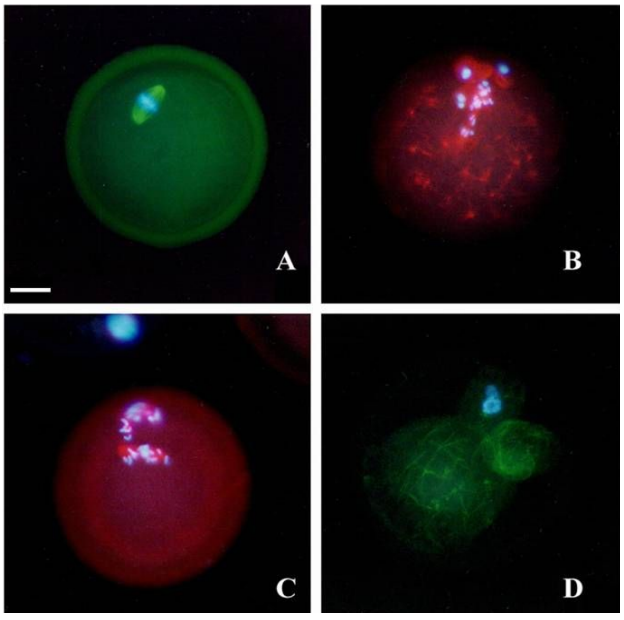

Fig. 2. Fluorescent microphotographs of chromosome alignment and spindle formation in the mouse fresh ovulated and the aged oocytes. A, A spindle assembly with aligned chromosomes. B and C, A spindle assembly with disaligned chromosomes. D, Abnormal microtubules in the cytoplasm of an oocyte. Bar indicates 10 ㎛.

Table 1. Percentages of the spindles with misaligned chromosomes in the mouse fresh ovulated and the post-ovulatory aged oocytes

Groups of oocytes	Post hCG injection	No. of oocytes	No. of spindles with misaligned chromosomes (%)
Fresh ovulated	16 hrs	51	4 (7.8)[a]
In vivo aged	24 hrs	49	6 (12.2)
	48 hrs	53	21 (39.6)[b]
In vitro aged	24 hrs	47	10 (21.3)
	48 hrs	45	32 (71.1)[b]

[a,b] Values with different superscripts differ significantly ($p<0.01$, Fisher's exact test).

Table 2. Alterations of the spindle lengths and widths in the mouse fresh ovulated and the post-ovulatory aged oocytes

Groups of oocytes	Post hCG injection	No. of oocytes	Spindle length	Spindle width
Fresh ovulated	16 hrs	51	24.9 ± 3.8[a]	15.6 ± 0.6[a]
In vivo aged	24 hrs	49	26.8 ± 2.5	16.0 ± 2.1
	48 hrs	53	31.4 ± 3.0[b]	13.2 ± 1.2[b]
In vitro aged	24 hrs	47	25.9 ± 2.3	14.8 ± 2.7
	48 hrs	45	35.7 ± 4.7[b]	11.7 ± 1.7[b]

Spindle lengths and widths were presented as mean ± SD (㎛).
[a,b] Values with different superscripts differ significantly ($p<0.01$, Student's *t*-test).

(Fig. 2B, C). Percentages of misaligned chromosomes in the aged oocytes were presented in Table 1. Only about 8% of the fresh ovulated oocytes had misaligned chromosomes in their metaphase plate. In the *in vivo* and the *in vitro* aged oocytes, the incidences of spindle with misaligned chromosomes were increased according to the aging time. The incidence of misaligned chromosomes was increased to 71% in the aged oocytes *in vitro* for 48 hours.

Measurement of the pole-to-pole distance and width of spindles in fresh ovulated showed that the average spindle length and width were 24.9 ± 3.8 ㎛ and 15.6 ± 0.6 ㎛, respectively. As shown in Table 2, the spindle length was increased and spindle width was decreased in the aged oocytes *in vitro* for 48 hours.

3. The Distribution of Acetylated Microtubules in the Fresh Ovulated and the Aged Oocytes

Acetylated tubulin was found at the poles through weaker spindle in the fresh ovulated oocytes. In the aged oocytes, acetylated tubulin staining intensity showed stronger than

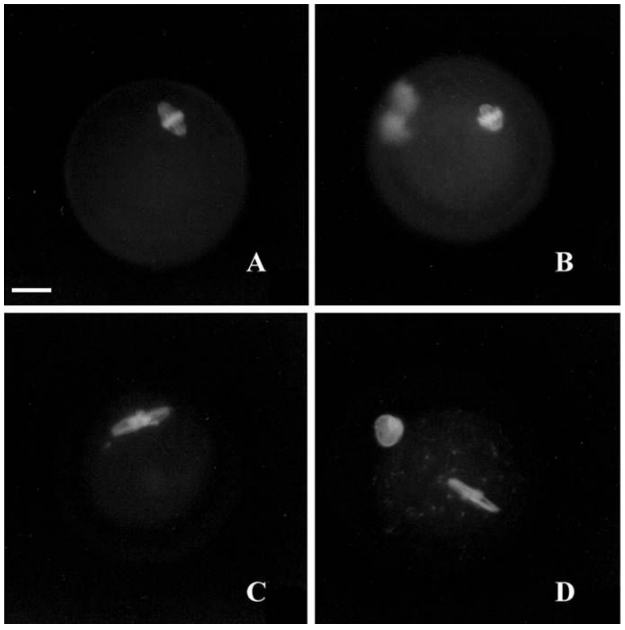

Fig. 3. Fluorescent microphotographs of the acetylated microtubules in the mouse fresh ovulated and the aged oocytes. A and B, Acetylated microtubules (green color) and chromosomes (blue color) in a fresh ovulated oocyte. C and D, Acetylated microtubules and chromosomes in an aged oocyte. Bar indicates 10 ㎛.

that in the fresh ovulated oocytes (Fig. 3). The cytoplasmic MTOCs in the aged oocytes were localized with the antibody to acetylated tubulin. On the contrary, it was not observed in the fresh ovulated oocytes.

DISCUSSION

The typical features of the microtubles in the fresh ovulated oocytes observed in this study are consistent with other observations by light and electron microscopy (Sato & Blandeau 1979; Szollosi et al., 1972) and by immunocytochemistry (Schatten et al., 1985; Eichenlaub-Ritter et al., 1986). This study also showed similar results of previous reports. Szollosi (1971) demonstrated structural alterations in the spindle during postovulatory ageing of the mouse oocytes in the fallopian tube. Webb et al. (1986) also observed the movement of the spindle from the periphery to the center and later its disruption of the spindle in the mouse aged oocytes. The change of average spindle length in the aged oocytes compared to the fresh ovulated oocytes in the mouse was reported (Eichenlaub-Ritter et al., 1986). They suggested that the gradual decrease in the critical concentration of tubulin in the aged oocytes to induce more microtubule polymerization away from the main spindle body. Unexpectedly, in this study, the spindle length of mouse oocytes was increased during post-ovulatory aging of mouse oocytes. This discrepancy was due to the different methods of microtubule stabilizing, permeabilization and fixation for immunocytochemistry. However, the overall results of misaligned chromosomes and disorganized spindle in the aged oocytes coincide with previous reports. Kim et al. (1996) observed that the pole-to-pole distance in the spindle of porcine oocytes was not changed during post-ovulatory aging using almost same methods. It may explain that the alteration pattern of spindle structures in the oocytes during aging is different mechanism by the species variation.

Schatten et al. (1988) reported that the mouse oocytes exhibited a specific sequence of microtubule acetylation throughout meiosis, fertilization and early embryonic development. They pronounced the post-translational modification is essential for development, acetylation of tubulin might contribute to cytoskeletal stability during development and the appearance of acetylated microtubules is a valuable marker for the presence of stable array. In this study, acetylated tubulin was detected in the spindle of fresh ovulated oocytes, and the staining intensity of acetylated tubulin was stronger in the aged oocytes than the fresh ovulated oocytes. The aged oocytes did not activated, thus dynamical rearrangement of microtubules assembly was not occurred. In the aged oocytes, static microtubules assembly may be maintained long time in the spindle structure. The static microtubules appear to be acetylated and the dynamic newly assembling ones do not. The post-ovulatory aged oocytes had more acetylated tubulins compared to the fresh ovulated oocytes in this study. The over static status of microtubules with acetylated tubulin may affect the chromosomal segregation during the 2nd meiosis after fertilization, and consequently it may cause the chromosomal abnormality in the embryo. One of major targets of acetylation is histone proteins of nucleic acids in the cell. Histone acetylation can alter interaction of histones and DNA, and regulate various gene expressions. Huang et al (2007) reported that raising the level of histone acetylation by trichostatin A accelerated the progression of post-ovulatory aging in mouse oocytes.

Recently, several molecules were reported as modulators of microtubules and spindle structure. Decline of MAD2 transcripts in aged oocytes increased frequencies of aneuploidy in mouse (Steuerwald et al., 2005) and porcine (Ma et al., 2005). In porcine oocytes, aging reduced expression of spindle checkpoint protein of MAD2 and antiapoptotic protein of BCL2, and MAP kinase activity. These events may cause abnormal sister chromatid segregation in meiosis and apoptosis in oocytes maturation and embryo development (Ma et al., 2005). It has been shown that BRCA1 of a tumor suppressor could regulate meiotic spindle assembly and checkpoint, implying a relationship between BRCA1 deficiency and aneuploid embryos (Xiong et al., 2008). In

maturation of mouse oocytes, Zap70 protein is synthesized MI stage and necessary for MI-MII transition. The amount of Zap 70 may decrease by aging and be related with alteration of MAPKs activity and spindle formation (Kim et al., 2009). It may be valuable to delay the progress of post-ovulating aging for preserve the normal developmental potency in specific conditions such as nuclear transfer and *in vitro* maturation of oocytes.

The present study indicated that there are dynamic alterations in microtubules assembly and spindle structure during post-ovulatory aging of mouse oocytes. The alteration of spindle formation and chromosomes alignment substantiates the poor development and the increase of disorders from the post-ovulatory aged oocytes. It might be important to fertilize on time in ovulated oocytes for the developmental competence of embryos with normal karyotypes. Non-invasive assessment of spindle structure and chromosome alignment in the ovulated oocytes may be a useful method of estimating the predisposition to aneuploid embryos in other mammals also, and especially in human oocytes.

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