Journal of Reproduction and Development, Vol. 59, No 3, 2013

—Original Article—

Nicotinamide: a Class III HDACi Delays *In Vitro* Aging of Mouse Oocytes

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Abstract. Postovulatory mammalian oocyte developmental potential decreases with aging *in vivo* and *in vitro*. Aging oocytes typically show cellular fragmentation and chromosome scattering with an abnormally shaped spindle over time. Previously, it was shown that histone acetylation in the mouse oocyte increased during aging and that treatment with trichostatin A (TSA), an inhibitor for class I and II histone deacetylases (HDACs), enhanced the acetylation, that is, aging. In this study, we examined the effect of nicotinamide (NAM), an inhibitor for class III HDACs, on *in vitro* aging of mouse oocytes as well as TSA. We found that treatment with NAM significantly inhibited cellular fragmentation, spindle elongation and astral microtubules up to 48 h of culture. Although presence of TSA partially inhibited cellular fragmentation and spindle elongation up to 36 h of culture, treatment with TSA induced chromosome scattering at 24 h of culture and more severe cellular fragmentation at 48 h of culture. Further, we found that α-tubulin, a nonhistone protein, increased acetylation during aging, suggesting that not only histone but nonhistone protein acetylation may also increase with oocyte aging. Thus, these data indicate that protein acetylation is abnormally regulated in aging oocytes, which are associated with a variety of aging phenotypes, and that class I/II and class III HDACs may play distinct roles in aging oocytes.

Key words: Astral microtubules, Cellular fragmentation, Chromosome scattering, Histone deacetylase, Nicotinamide, Oocyte aging, Spindle elongation, Trichostatin A, Tubulin acetylation

(**J. Reprod. Dev. 59:** 238–244, 2013)

After ovulation or *in vitro* maturation, mammalian oocytes are arrested at meiotic metaphase II (MII) until they are activated by penetrating spermatozoa or artificial stimuli. Mammalian oocytes have a limited time for fertilization after ovulation. The window for optimal fertilization differs in different species, and it has been determined that mouse, rat, and monkey and human oocytes exhibit the most potential 8–12 h, 12–14 h and <24 h after ovulation, respectively [1, 2]. If not fertilized or activated within that time, mature oocytes progressively undergo a time-dependent process of aging, which leads to a decrease in the potential for fertilization and embryo development [3]. In addition, aged oocytes show the phenomenon of a high proportion of cellular fragmentation and cell death [4, 5]. However, the precise mechanisms involved in these cellular fragmentation phenomena in oocyte aging are not yet well understood. In the mouse, following ovulation, the fragmentation of unfertilized mature oocytes has been viewed as a manifestation of apoptosis, or programmed cell death [6−9], and is enhanced by a variety of factors, including oxidative stress, diabetes and specific gene mutations [10−14].

Many reagents have been reported to prevent abnormal features after aging in oocytes. The reports have shown that age-associated cellular fragmentation is partially prevented by incubation with β-mercaptoethanol at 24 h after oocyte collection. DTT increased the potential of aged mouse oocytes to develop to the blastocyst stage [15, 16]. Nitric oxide delays oocyte aging and improves the integrity of the microtubular spindle apparatus in mice [17]. Recently, caffeine and MG132 have also been implicated in preventing aging of mouse oocytes [18].

Histone acetylation by histone acetyltransferases (HATs) and histone deacetylation by histone deacetylases (HDACs) play important roles in various cellular functions for opposing activities that modulate gene expression through chromatin modification. HDACs are divided into five categories: class I (HDAC 1–3 and 8), class IIa (HDAC 4, 5, 7 and 9), class IIb (HDAC 6 and 10), class III (SIRT 1–7) and class IV (HDAC 11) [19]. The structurally distinct class III HDACs, which contain the family of sirtuins, comprise a unique class of nicotinamide adenine dinucleotide (NAD+)-dependent deacetylases [20] that are involved in diverse biological functions such as metabolism, cell division and aging [21]. Nicotinamide (NAM) is known as a class III HDAC inhibitor, a noncompetitive inhibitor of sirtuin [22]. Budding

Received: November 15, 2012
Accepted: January 4, 2013
Published online in J-STAGE: March 10, 2013

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yeast grown in the presence of added NAM causes defects in Sir2-mediated transcriptional silencing, increasing rDNA recombination, and a significantly shorter lifespan [23]. Depletion of nicotinamide by PNC1 extends longevity and prevents NAM-induced inhibition of telomeric and rDNA silencing in yeast [24, 25]. Nicotinamide inhibits p53 deacetylation by Sir2α upon DNA damage in mouse embryonic fibroblast cells [26].

Inhibition of other classes of HDACs has also been shown to affect phenotypes associated with oocyte aging. The acetylation levels of lysine 14 on histone H3 (H3K14) and lysine 8 and 12 on histone H4 (H4K8/K12) in mouse oocytes increase during aging of oocytes [27]. Treatment of oocytes during aging with trichostatin A (TSA), an inhibitor of HDACs, increases acetylation of H3K14 and H4K8/K12, suggesting that treatment of oocytes with HDAC inhibitors (HDACis) may accelerate the progression of oocyte aging [27]. In contrast, inhibition of HDACs by TSA may instead reduce the percentage of cellular fragmentation in *in vitro* aging of pig oocytes [28]. These reports suggest that the acetylation statuses of proteins in oocytes are positively or negatively associated with oocyte aging.

In the present study, we focus on the effects of HDACis on the morphologies, chromosome alignment and spindle morphologies as well as acetylation status of α-tubulin, a nonhistone protein in aging oocytes.

Materials and Methods

Animals

We used female B6D2F1 mice (6–8 weeks of age) purchased from Shizuoka Laboratory Animal Center (Hamamatsu, Japan). Animal care and experiments were in accordance with the Guiding Principles for the Care and Use of Laboratory Animals in a Kinki University Animal Facility.

Oocyte collection

We used *in vitro*-aged oocytes that were superovulated by injection of pregnant mare's serum gonadotropin (PMSG) followed 48 h later by injection of human chorionic gonadotropin (hCG). Oocytes were collected in an M2 medium at 15 h after hCG injection, and cumulus cells were removed with M2 containing 0.1% hyaluronidase. After 3–4 rinses, cumulus-free oocytes were cultured in KSOM (Millipore) medium for the experiment.

In vitro aging and assessment of oocyte abnormalities

For *in vitro* aging, some oocytes were directly used for the fresh experimental group (MII), and oocytes were cultured in KSOM medium supplemented with or without of 20 mM nicotinamide (NAM; Sigma) or 500 nM trichostatin A (TSA; Sigma). The oocytes were then cultured at 37 C in 5% CO_2 in air for 0, 24, 36 and 48 h. At different time intervals of *in vitro* aging, the oocytes were subjected to morphological examination, Western blot analysis and immunostaining with α-tubulin, acetylated α-tubulin, and other aging parameters such as spindle elongation and chromosome scattering. At least 20 oocytes were evaluated at each replication at least three times.

SDS-Page and Western blotting

Total protein was collected from 30 oocytes from each group, which were lysed in SDS sample buffer and boiled at 100 C for 5 min and loaded onto 10% SDS-polyacrylamide gels. Total proteins were separated by SDS-PAGE and electrophoretically transferred to a membrane, which was then blocked at 1 h and incubated with a 1:5000 dilution of rabbit monoclonal anti-α-tubulin (T3526; Sigma) and a 1:10000 dilution of mouse monoclonal anti-acetylated α-Tubulin (T7451; Sigma) overnight at 4 C. The membranes were then washed three times in PBS-Tween, and membranes were incubated in an anti-mouse secondary antibody or an anti-rabbit secondary antibody for 1 h at room temperature. The membranes (NH 1703; Amersham Hybond™-P) were thoroughly washed with PBS-Tween three times and then processed with an ECL (4622840; Amersham™ ECL™ prime Western Blotting Detection Reagent) detection system. For quantitative analysis of α-tubulin and acetylated α-tubulin levels, Western blot images were subjected to densitometric analysis using the ImageJ software from the National Institutes of Health (http://rsb.info.nih.gov/ij/) (USA).

Immunofluorescence

MII oocytes at 0 h (15 h after hCG injection), 12 h (27 h after hCG injection), 24 h (39 h after hCG injection) and 36 h (51 h after hCG injection), treated with or without NAM or TSA, were washed with 0.1% polyvinyl alcohol (PVA) containing PBS (PBS-PVA) and then fixed in 4% w/v paraformaldehyde at room temperature for 30 min. After washing in PBS-PVA, the oocytes were incubated overnight in PBS containing 1% bovine serum albumin (BSA) and 0.1% Triton X-100 at 4 C. Then the oocytes were washed three times with PBS 0.1% BSA and incubated with the primary antibodies, a 1:200 dilution of a rabbit monoclonal anti-α-tubulin (T3526; Sigma) and a 1:1000 dilution of a mouse monoclonal anti-acetylated α-tubulin (T7451, Sigma), for 2 h at room temperature. After washing three times with PBS-0.1% BSA, the oocytes were incubated with the secondary antibodies, 1:200 goat anti-mouse or 1:200 chicken anti-rabbit antibodies, for 1 h at room temperature. After washing three times with PBS-0.1% BSA, the DNA was visualized by DAPI staining. The oocytes were mounted on glass slides with a drop of fluorescent mounting medium, and then covered by a glass cover slip. We observed each cytoplasm and spindle by fluorescence microscopy in aging oocytes treated with NAM and TSA.

Statistical analysis

The data were basically compared using chi-square test analysis with Yates correction for continuity. The data of normal and abnormal morphologies were analyzed by Welch's *t* test. A value of $P<0.01$ or 0.05 was considered to be statistically significant.

Results

Effects of nicotinamide or trichostatin A on aging oocytes

To investigate how NAM or TSA inhibitors for HDAC I/II and III respectively affect abnormal morphologies during oocyte aging, we observed aging oocytes for up to 48 h in a culture medium treated with or without of NAM or TSA following oocyte collection. We classified abnormal morphologies of aging oocytes into three categories, cellular fragmentation, 2-cell-like structure and normal oocyte morphology, as we previously reported [29]. Untreated aging oocytes started to

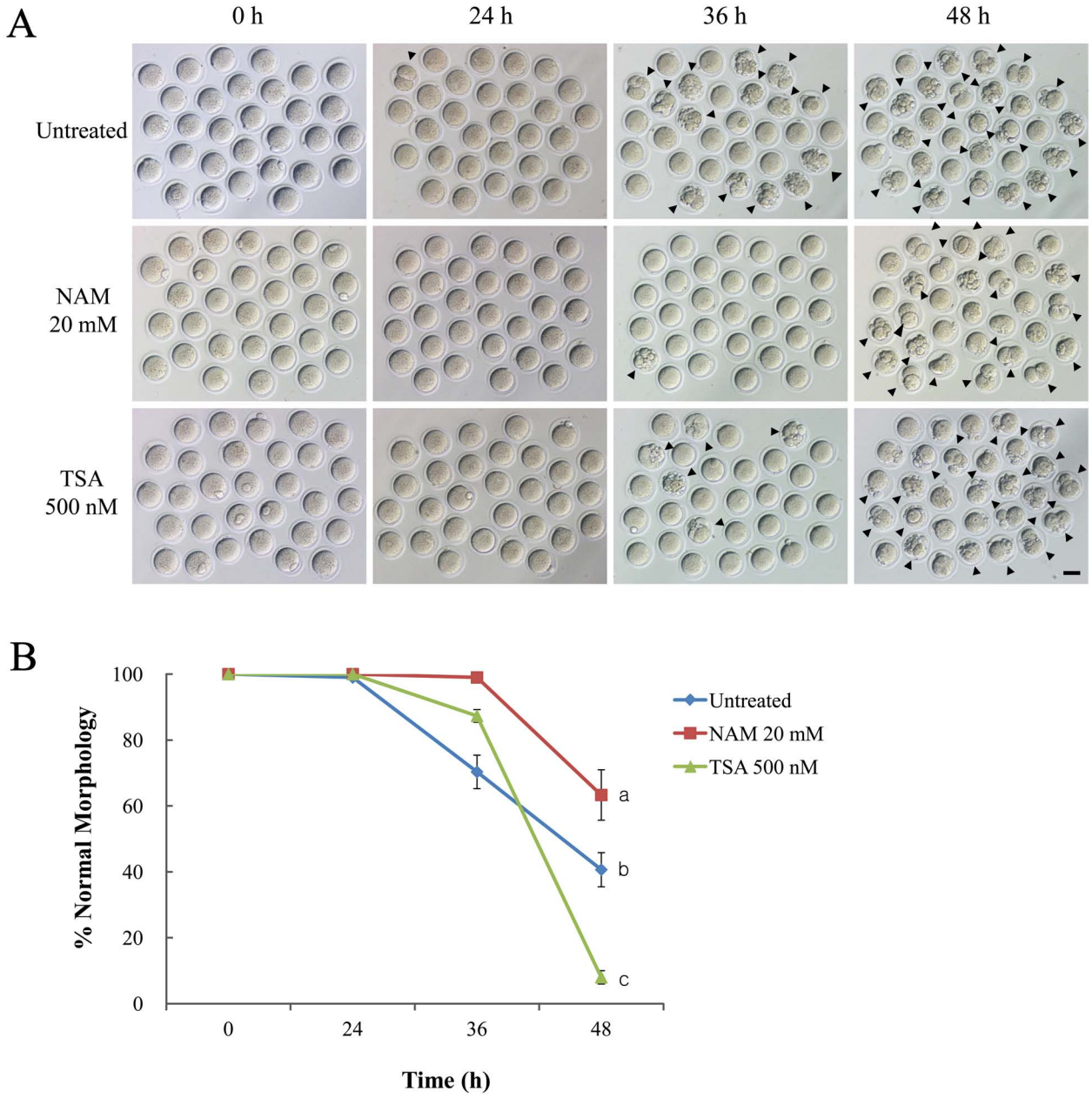

Fig. 1. Morphological changes of postovulatory aging oocytes treated with NAM, or TSA at 0, 24, 36 and 48 h after oocyte collection. A: Although some oocytes started to show abnormal morphologies at 36 h, such as cellular fragmentation and 2-cell-like structure (arrowheads: abnormal oocytes), more oocytes treated with NAM or TSA continued to show normal morphology. However, at 48 h, most oocytes showed fragmentation except those treated with NAM. Bar = 100 μm. B: Summary of quantification of oocytes with normal morphology. Data are given as mean % ± SEM.

exhibit abnormal morphologies at 36 h, and by 48 h, more than half of the aging oocytes had shown abnormal morphologies (Fig. 1A, B). Although treatment with NAM or TSA reduced the frequency of abnormal oocyte at 36 h, treatment with NAM significantly inhibited the abnormal morphologies in aging oocytes even at 48 h (Fig. 1A, B). Treatment with TSA at 48 h resulted in a greater number of fragmented oocytes than untreated aging oocytes (Fig. 1A, B). Thus, HDACis affected oocyte fragmentation associated with aging, and the inhibitor of Class III HDAC more effectively suppressed oocyte fragmentation. Therefore, regulation of protein acetylation may play important roles in oocyte aging.

Acetylation of α-tubulin increases during oocyte aging

Aging oocytes increased histone acetylation [27]. However, it is not known how protein acetylation is generally associated with oocyte aging. α-Tubulin, a nonhistone protein, is a well-known

representative substrate of HDACs in the cytoplasm. Therefore, we focused on the acetylation status of α-tubulin during aging in oocytes. Compared with the freshly collected oocytes (0 h), the amounts of both acetylated α-tubulin (Ac α-tubulin) and α-tubulin gradually increased in the oocytes during aging until 24 h, with the amount of Ac α-tubulin continuing to accumulate after this time point (Fig. 2A–C). It should be noted that at 36 h, aged oocytes showed astral microtubules that were not always associated with Ac α-tubulin (Fig. 2A). Thus, these data indicate that the amount of α-tubulin and its acetylation status change along with oocyte aging.

Treatment with NAM and TSA affect acetylation status of α-tubulin in aging oocyte

Next, we examined effects of HDACi treatment on the acetylation status of α-tubulin in oocytes during aging. Treatment with TSA dramatically increased Ac α-tubulin in all the oocytes compared with untreated aging oocytes at 36 h (Fig. 3A–C). In contrast, although treatment with NAM resulted in similar amounts of Ac α-tubulin and α-tubulin at 36 h, treatment with NAM maintained a lower level of Ac α-tubulin and α-tubulin in cytoplasm compared with control aging oocytes at 36 h (Fig. 3A–C). Further, astral microtubules, a symptom of oocytes aging, were observed in most untreated aging oocytes and TSA-treated oocytes at 36 h (Table 1). On the other hand, in the presence of NAM, the number of oocytes with astral microtubules was significantly reduced at 36 h in cytoplasm (Table 1). Thus, NAM treatment strongly suppressed production of abnormal microtubule structures during aging. Together, these results suggest that oocyte aging increases the amount of Ac α-tubulin and α-tubulin, which may contribute to the production of astral microtubules.

Treatment with NAM or TSA impacts spindle morphology during oocyte aging

Aged oocytes have abnormally elongated spindles [18, 29]. Therefore, we next focused on the effects of NAM or TSA on spindle morphology in aging oocytes. Although at 24 h most of the aged oocytes displayed an irregularly shaped spindle, treatment with NAM resulted in a significant reduction in spindle elongation (Fig. 4A, B). It should be noted that treatment with TSA led to apparent prevention of spindle elongation and also led to a loss of microtubules from the spindle (Fig. 4A). Taken together, inhibition of HDAC affects stability of spindle morphology associated with oocyte aging.

Discussion

Cellular fragmentation commonly occurs in postovulatory aging oocytes concomitantly with loss of developmental potential. The oocyte fragmentation *in vitro* is dependent upon the functional expression of several genes comprising the evolutionarily conserved apoptotic cell death program [30, 31].

In this study, we revealed that treatment of aging oocytes with HDACis, TSA and NAM, affects the cellular fragmentation and abnormal spindle morphology of aging oocytes. It is known that TSA inhibits classes I and IIa/b HDACs and that NAM can inhibit SIRT 1 and 2, which are class III HDACs [32]. SIRT 1 regulates p53 acetylation and p53-dependent apoptosis in response to DNA damage and oxidative stresses [33]. SIRT 2 is a cytoplasmic protein and has a role in tubulin deacetylation [34]. Recently, it has been reported that NAM and TSA induce apoptosis in neural stem cells via distinct molecular mechanisms [35], suggesting that different signaling pathways based on inhibition of different HDACs could share one phenotype. It was reported that all sirtuin genes (Sirt 1-7) are expressed in ovulated oocytes [36]. We showed that treatment with NAM inhibits the cellular fragmentation and 2-cell-like structures until 36 h (51 h after hCG injection) after oocyte collection. These data suggest that NAM strongly inhibits abnormal phenotypes in progressive oocyte aging and that TSA shows weak and limited inhibition.

Some specific residues of histone such as lysine 14 on histone H3 (H3K14) and lysine 8 and 12 on histone H4 (H4K8/K12) are associated with oocyte aging [27]. In this study, we examined the level of Ac α-tubulin to elucidate the role of acetylation of non-histone proteins during oocyte aging. Mouse oocytes exhibit microtubule acetylation after fertilization and early development [37]. The acetylation of α-tubulin contributes to cytoskeletal stability during development, and the appearance of acetylated microtubules is a valuable marker for the presence of stable arrays [38, 39]. α-Tubulin is deacetylated by HDAC6 [40] and the NAD+ dependent histone deacetylase SIRT2 [41] and acetylated by MEC-17 [42]. In this study, we found that the levels of both Ac α-tubulin and α-tubulin increased in the aging oocytes. Our report first described dynamics of Ac α-tubulin and α-tubulin in aging oocytes. The mechanism underlying accumulation of α-tubulin in aging oocytes is not known, but acetylation status and/or localization of α-tubulin may contribute to the stability and turnover of α-tubulin. Actually, more acetylation of α-tubulin by TSA treatment resulted in a larger accumulation of α-tubulin in the cytoplasm, but led to a loss of microtubules from the spindle at 36 h of aging. Regardless of the amount of α-tubulin, the ratio of Ac α-tubulin increased during oocyte aging. Thus, increasing acetylation in both histone and nonhistone protein occurs during oocyte aging, suggesting that aging oocytes lose the precise regulation of protein acetylation by HATs and HDACs.

Murine MII oocytes contain two microtubule-containing structures: the meiotic spindles and a dozen cytoplasmic microtubules (astral microtubules) [43]. The meiotic spindles are crucial for the proper alignment and separation of the chromosomes during meiosis, whereas the cytoplasmic astral microtubules are responsible for pronuclear apposition following sperm incorporation [44–46]. It should be noted that the α-tubulin acetylation is dynamically assembled astral microtubules emanating from the spindle into the cytoplasm at 36 h. However, treatment with NAM could suppress formation of astral microtubules in aging oocytes. These results suggest that the assembled astral microtubules in the cytoplasm associated with aging are regulated by class III HDACs. NAM was able to minimize acetylation of α-tubulin until 36 h in contrast to untreated aging oocytes.

Next, we examined the spindle morphology in aging oocytes. It is reported that when a freshly ovulated oocyte is aged *in vitro*, it leads to misaligned chromosomes or dispersed, elongated or completely disrupted spindles, which are associated with poor developmental potential in embryos [25]. We found that most aged oocytes appear to display spindle elongation at 24 h. On the other hand, most aging oocytes treated with NAM showed significantly inhibited spindle

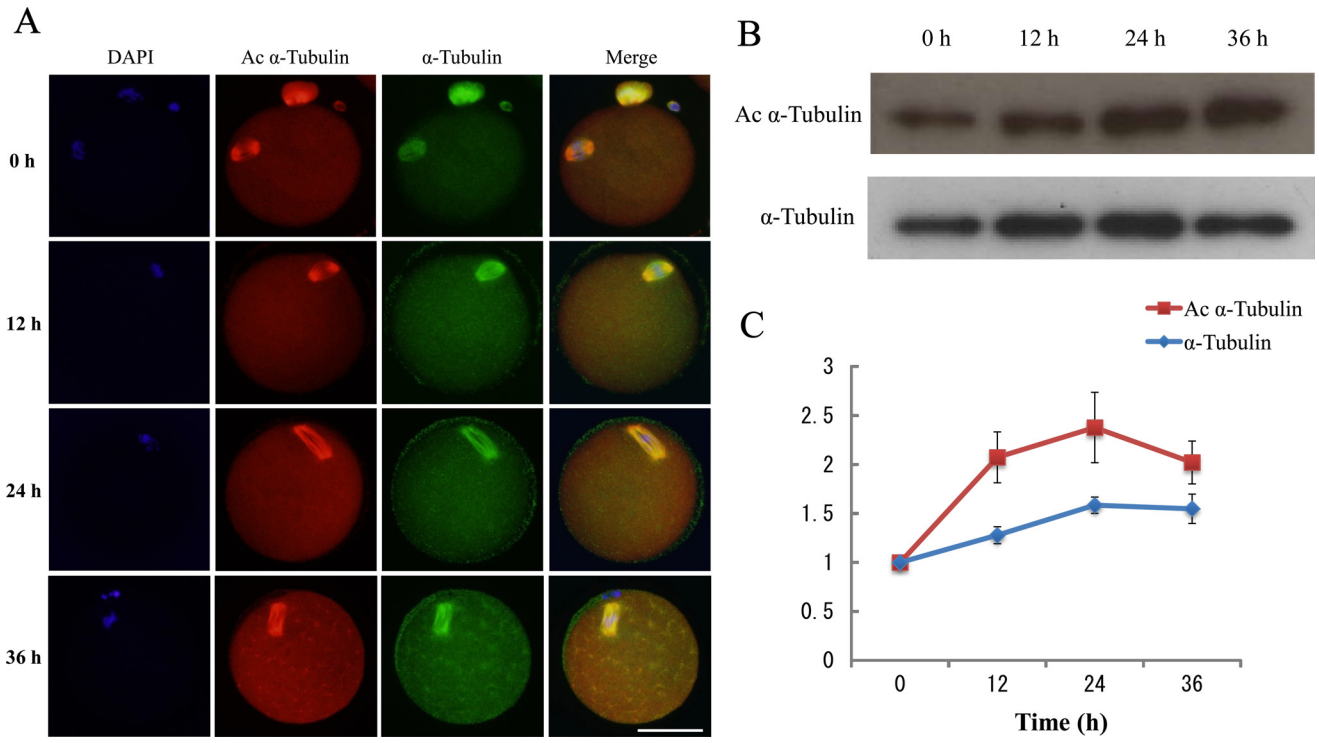

Fig. 2. Acetylated α-tubulin (Ac α-tubulin) and α-tubulin were increased in aging oocytes. A: Immunostaining of aging oocytes with anti-Ac α-tubulin and anti-α-tubulin antibodies revealed that during oocyte aging, the level of Ac α-tubulin and α-tubulin was increased until 24 h and then decreased at 36 h. B: Consistent with the results of immunostaining, Western blot analysis confirmed that the level of Ac α-tubulin and α-tubulin increased until 24 h in aging oocytes but started to reduce at 36 h. Elongated spindles (24 h) and astral microtubules (36 h) were observed. C: Summary of Western blot quantification. Data are given as mean % ± SEM. Ac α-tubulin showed a greater increase than α-tubulin itself.

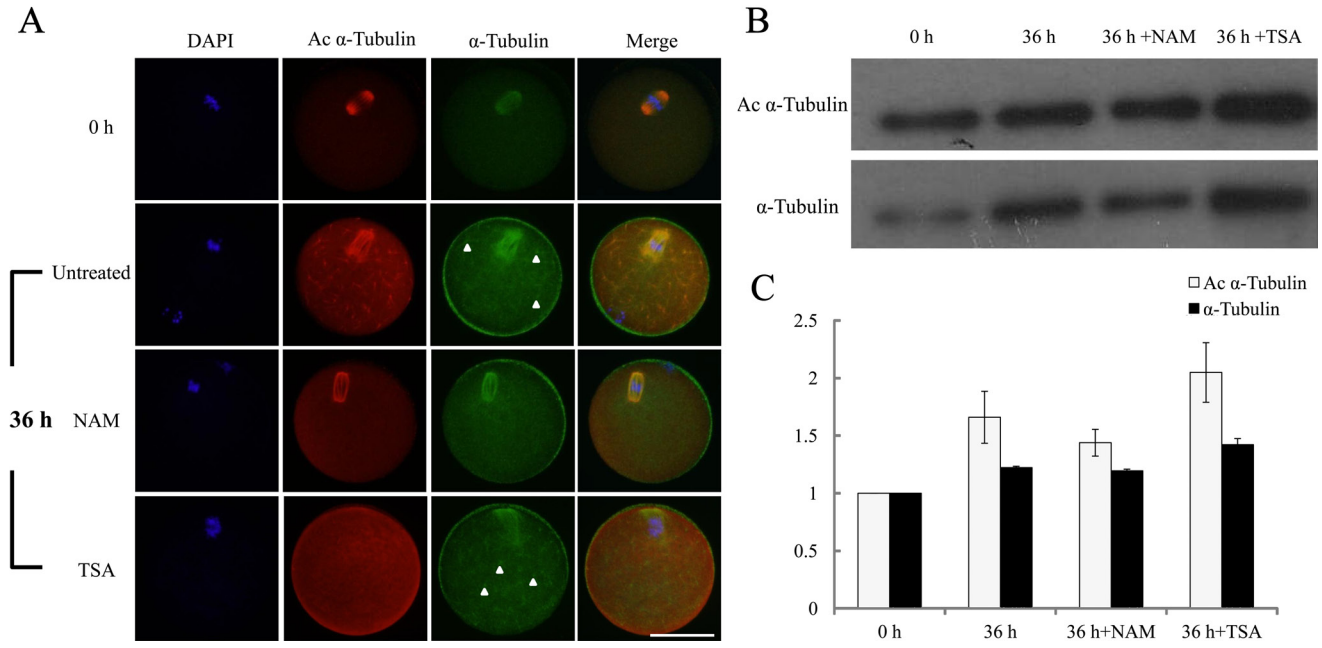

Fig. 3. Impact of NAM and TSA on status of Ac α-tubulin and on abnormal structures of α-tubulin at 36 h after oocyte collection. A: Immunostaining of aging oocytes with anti-Ac α-tubulin and anti-α-tubulin antibodies at 36 h revealed that NAM treatment suppressed the increase of acetylation in α-tubulin and maintained normal spindle morphologies and appearance of astral microtubule structures (arrowheads). In contrast, TSA treatment induced more acetylation and severe spindle degeneration in microtubules. Bar = 100 μm. B: Consistent with the results of immunostaining, Western blot analysis confirmed that the level of Ac α-tubulin and α-tubulin increased until 36 h in aging oocytes after TSA treatment but not in NAM-treated aging oocytes compared with untreated aging oocytes. C: Summary of Western blot quantification. Data are given as mean % ± SEM. TSA treatment showed a greater increase than α-tubulin.

Table 1. Oocytes with astral microtubules after 36 h of aging

Treatment	No. oocyte	No. of oocytes with astral microtubules (%)
Fresh MII	75	1 (1)[a]
Untreated-36 h	75	74 (99)[b]
20 mM NAM-36 h	83	16 (19)[c]
500 nM TSA-36 h	77	39 (51)[d]

[a, b, c, d] $P<0.01$; n=5.

elongation. Our results provide evidence that increased numbers of normal spindles and chromosomes are formed because of changes in the levels of Ac α-tubulin, which regulates the astral microtubules in the cytoplasm. The astral microtubules might be from the unstable spindle microtubules emanating into the cytoplasm because of oocyte aging and cellular fragmentation in aging oocytes.

In conclusion, inhibition of the class I/II and especially III HDACs delays aging phenotypes. Further, the level of Ac α-tubulin and α-tubulin increased in the process of oocyte aging, which is also suppressed by NAM treatment. Thus, NAM could inhibit cellular fragmentation and spindle elongation and minimize acetylation of α-tubulin in oocyte aging. Finally, our findings present the first demonstration of the involvement of Class I/II and III HDACs in aging phenotypes while providing insight into the mechanism underlying oocyte aging and prevention of abnormal phenotypes in aged oocytes.

Acknowledgements

We gratefully acknowledge discussions with Drs A Ito, J Kimura, K Matsubara and T Castranio. This work was supported by the PRESTO (Precursory Research for Embryonic Science and Technology) program of the Japan Science and Technology Agency and a Grant-in-Aid for Scientific Research (KAKENHI) from the Japan Society for the Promotion of Science (grant number 23580416, to SK).

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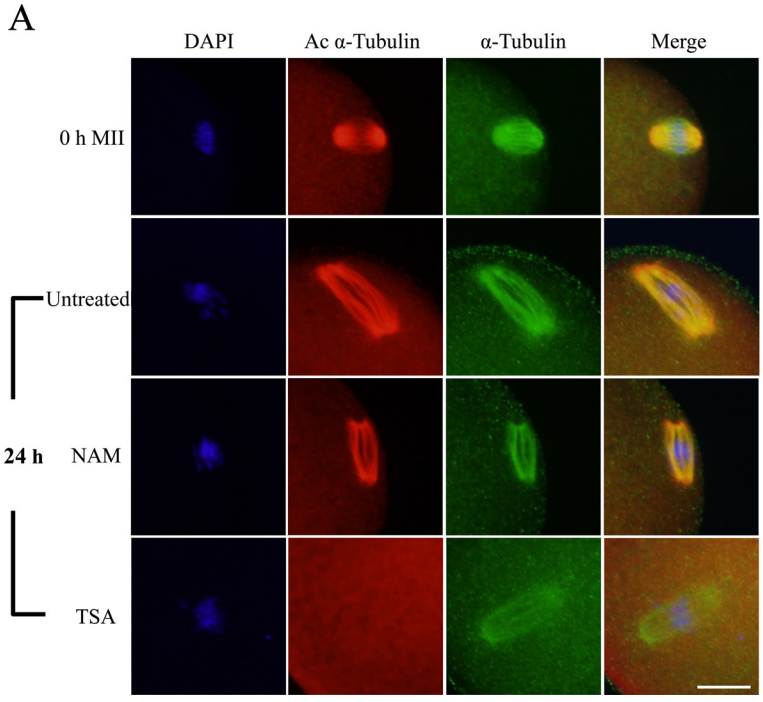

Fig. 4. Impact of NAM and TSA treatment on spindle elongation at 24 h. A: Immunostaining of aging oocytes with anti-Ac α-tubulin and anti-α-tubulin antibodies at 24 h revealed that NAM treatment maintained compact spindle formation at MII but that TSA treatment partially prevented spindle elongation associated with microtubule loss in spindles. Bar = 100 μm. B: Summary of quantification of the length of spindles compared with freshly collected oocytes (0 h).

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