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Effect of trichostatin A on fertilization and embryo development during extended culture of mouse oocyte

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Summary

We performed this study to investigate the effect of histone deacetylase inhibition during extended culture of *in vitro* matured mouse oocytes. *In vitro* matured mouse (BDF1) oocytes were cultured *in vitro* for 6, 12, and 24 h, respectively, and then inseminated. During *in vitro* culture for 6 and 12 h, two doses of trichostatin A (TSA), a histone deacetylase inhibitor, were added (100 nM and 500 nM) to the culture medium and the oocytes were then inseminated. During the 24-h *in vitro* culture, two doses of TSA were added (100 nM and 500 nM) to the medium and the oocytes were activated with 10 mM $SrCl_2$. After the 6-h culture, the fertilization rate was similar to that of the control group, but the blastocyst formation rate was significantly decreased. After the 12-h culture, both the fertilization and blastocyst formation rates were significantly decreased. After the 24-h culture, total fertilization failure occurred. In the oocytes cultured for 6 and 12 h, the fertilization and blastocyst formation rates did not differ between the TSA-supplemented and control groups. Although extended culture of the mouse oocytes significantly affected their fertilization and embryo development, TSA supplementation did not overcome their decreased developmental potential.

Keywords: Extended culture, Histone deacetylase, *In vitro* maturation, Oocyte, Trichostatin A

Introduction

It has been well established that fragmentation increases and fertilization and embryo development rate decrease after prolonged *in vitro* culture of oocytes (Tatone *et al.*, 2008; Miao *et al.*, 2009). The cellular and molecular mechanisms of oocyte aging have been studied, and several investigators have attempted to delay the aging process by the supplementation of oocytes with various substances (Goud *et al.*, 2005; Rausell *et al.*, 2007).

Oocyte aging is associated with various functional changes such as a decrease in fertilization, polyspermy, digyny, parthenogenesis, chromosomal abnormalities, apoptosis, early extrusion of cortical granules, zona hardening, and a decrease in the activities of maturation promoting factor (MPF) and mitogen-activated protein kinase (MAPK) (Miao *et al.*, 2009). Mature oocytes in the metaphase II (MII) stage have a high MPF activity, which decreases with aging; a gradual age-related decrease in MPF activity is one of the causes of oocyte aging (Kikuchi *et al.*, 2000; Huang *et al.*, 2007).

Recently, the association between oocyte aging and epigenetic changes has been studied (Liang *et al.*, 2008). Histone acetylation is suggested as one of the major mechanisms of epigenetic regulation during the zygote stage and development after fertilization (Akiyama *et al.*, 2006; Endo *et al.*, 2006; Ma & Schultz, 2008). In oocytes, histone acetylation is significantly associated with the maturation of murine and porcine oocytes (Kim *et al.*, 2003; Akiyama *et al.*, 2004; Spinaci *et al.*, 2004; Endo *et al.*, 2005; Wang *et al.*, 2006; Bui

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et al., 2007; Petr *et al.*, 2009). The degree of histone acetylation depends on the balance between histone acetyltransferase and histone deacetylase (HDAC), which promote acetylation and deacetylation, respectively. In mammalian oocytes, histones H3 and H4 are highly acetylated during the germinal vesicle (GV) stage, and global histone deacetylation takes place via an HDAC-dependent mechanism during GV breakdown (GVBD) and metaphase I (MI). Acetylation temporarily increases during anaphase I (AI) and telophase I (TI) and finally decreases during the MII phase (Kim *et al.*, 2003; Wang *et al.*, 2006; Huang *et al.*, 2007). However, in porcine oocytes, the total HDAC activity is maintained throughout meiotic maturation, and thus HDACs are inactive in GV oocytes (Endo *et al.*, 2008).

In mature murine oocytes, histone acetylation increases with age, and this increase has been observed in both *in vivo* and *in vitro* aged oocytes (Huang *et al.*, 2007). HDAC inhibition increases histone acetylation and the supplementation of murine oocyte cultures with an HDAC inhibitor can induce parthenogenesis, one of the oocyte-aging phenomena (Huang *et al.*, 2007). However, in porcine oocytes, the inhibition of HDAC during oocyte aging significantly reduces the percentage of fragmented oocytes and significantly increases the proportion of oocytes progressing to the blastocyst stage (Jeseta *et al.*, 2008). The objective of this study was to investigate the effect of HDAC inhibition during extended cultures of *in vitro* matured mouse oocytes.

Materials and methods

In vitro maturation of immature oocytes

Five- to 6-week-old female BDF1 mice (Orient Co.) were used in this experiment. Animal care and use were in accordance with the institutional guidelines established by the Animal Care and Use Committee of Seoul National University Bundang Hospital.

Female mice were injected with 7.5 IU equine chorionic gonadotropin (eCG; Sigma) and were killed 48 h later by cervical dislocation. Ovaries were collected in 1 ml of washing medium (modified mouse tubal fluid, mMTF) supplemented with 0.4% bovine serum albumin (BSA; Sigma). The cumulus-enclosed oocytes (CEOs), covered with compact cumulus cells, were collected by puncturing the antral follicles. Only CEOs with a uniform covering of cumulus cells were used in this study. A part of the immature oocytes was obtained 48 h after eCG priming and then matured *in vitro* using maturation medium for 17 to 18 h. The maturation medium consisted of a commercial TCM199 (Invitrogen) supplemented with 20% fetal bovine serum (FBS; Invitrogen) and recombinant FSH/hCG (75 mIU/ml and 0.5 IU/ml) (Serono). In all cultures, groups of up to 10 oocytes were placed in 50-μl microdrops of medium under mineral oil (Sigma) in 35 × 10-mm Petri dishes (Falcon; Becton Dickinson) and were placed at 37°C in humidified 5% CO_2 in air for 17 to 18 h. At the end of IVM, all CEOs were denuded completely by being treated with 85 IU/ml hyaluronidase (Sigma), and the nuclear maturation of the oocytes was assessed. The first polar body extrusion was used as the maturation criterion under an inverted microscope (×200).

In vitro aging of MII oocytes

Parts of the immature oocytes were subjected to IVM and IVF to clarify their developmental competence.

Experiment 1

Only matured oocytes with the first polar body were transferred to the fertilization medium (mMTF supplemented with 0.8% BSA) in which *in vitro* aging was induced for 6, 12, and 24 h each.

After *in vitro* aging, the oocytes were divided into four groups: intact, parthenogenetically activated, fragmented, and lysed state. Only the intact oocytes were fertilized *in vitro*. The epididymal spermatozoa were retrieved from the cauda epididymis of 8- to 10-week-old BDF1 mice, and the sperm suspensions were pre-incubated for 1.5 h in capacitation medium (mMTF supplemented with 0.8% BSA). The oocytes were then inseminated by sperms at a final dilution of 2 × 10^6/ml and incubated at 37°C in humidified 5% CO_2 in air. The inseminated oocytes were washed away from the sperms by gentle pipetting 4 h later and then placed in embryo maintenance medium (mMTF supplemented with 0.4% BSA). Fertilization was assessed by the formation of two cells on day 1 (the day after insemination). The cleaved embryos were transferred to new embryo maintenance medium and development to blastocyst was recorded on day 5 after insemination.

Experiment 2

The *in vitro* matured oocytes were transferred to microdrops of fertilization medium containing HDAC inhibitor trichostatin A (TSA, Sigma) with two different doses (100 nM, 500 nM) and then incubated for 6 or 12 h. Stock solutions of TSA were prepared by dissolving the compounds in DMSO at a concentration of 5 mM/ml (DMSO 0.1%) and stored at –20°C. These stock solutions were diluted in fertilization medium to produce the final two concentrations. The final concentrations of DMSO were less than 0.1% in each group. The control group did not contain TSA.

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Table 1 Effect of extended culture of *in vitro* matured mouse oocyte on fertilization and subsequent development

Time for extended culture (h)	GV	MII	After extended culture				Inseminated	2-cells (%)	Blastocyst (%)
			Intact	Spontaneously activated	Fragmented	Lysed			
0	86	49	–	–	–	–	49	37 (75.5)	14 (37.8)
6	53	32	32	0	0	0	32	22 (68.8)	1 (4.5)[a]
12	53	30	30	0	0	0	30	15 (50.0)[a]	0[a]
24	119	80	64	5	11	0	64	0[a]	0[a]

[a] $p < 0.05$ when compared with the 0 h extended culture group.

Both the fertilization and embryo culturing process were completely the same as those of Experiment 1.

Experiment 3

The oocytes were transferred to fertilization medium containing TSA with two different doses (100 nM, 500 nM) and then incubated for 24 h. We performed artificial parthenogenetic activation because the 24 h aging oocytes did not achieve fertilization using the normal insemination method.

Parthenogenetic activation. The *in vitro*-aged oocytes were incubated in activating media for varying durations at 37.5°C in a humidified atmosphere with 5% CO_2 in air. The activating media were Ca^{2+}-free M16 (NaCl adjusted to 6.437 g/l) supplemented with 10 mM concentrations of $SrCl_2$ (Sigma). The oocytes were cultured for 2.5 h in activating media. After $SrCl_2$ treatment, the oocytes were cultured in regular IVF medium. Six hours after the onset of activation treatment with $SrCl_2$, the oocytes were examined microscopically for evidence of activation. The oocytes were considered to be activated when each contained 1 (1PN) or 2 well developed pronuclei (2PN). The activated oocytes were transferred to the fertilization medium. Cleavage embryos were assessed by the formation of two cells on day 1 (the day after activation). The embryos were transferred to new embryo maintenance medium and development to blastocyst was recorded on day 5 after activation.

Statistical methodology

Data were analysed with MedCalc Software (ver. 6.10, Mariakerke, Belgium). The proportions were compared using the chi-squared test and were considered statistically significant when a p-value was <0.05.

Results

Experiment 1

To evaluate the effect of the extended culture of *in vitro* matured mouse oocytes on fertilization and subsequent development, we analysed oocyte quality and embryo development after 6, 12, and 24 h of *in vitro* culture (Table 1). After 6 h of *in vitro* culture, there were no activated, fragmented, or lysed oocytes, and the fertilization rate was not different from the control group. The blastocyst formation rate, however, was significantly decreased compared to that of the control group. After 12 h of culture, there were also no activated, fragmented, or lysed oocytes, but the fertilization and blastocyst formation rates both significantly decreased. Activated and fragmented oocytes were observed after 24 h of culture, and there were no fertilized oocytes after sperm insemination.

Experiment 2

There was no difference in the fertilization and blastocyst formation rates between the TSA supplementation groups and the control group during 6 h of extended culture (Table 2). Similarly, there was no difference in the fertilization and blastocyst formation rates between the TSH supplementation groups and the control group after 12 h of extended culture (Table 3).

Experiment 3

Parthenogenetic activation occurred after stimulation with $SrCl_2$ in both the TSH supplementation groups and the control group (Table 4). The 2-cell embryo development rate showed a decreasing trend in the TSA supplementation groups.

Discussion

In mammals, the window for optimal fertilization differs in different species and has been determined for the mouse (8–12 h), rat (12–14 h), rabbit (6–8 h), rhesus monkey, and human (24 h) (Miao *et al.*, 2009). If fertilization does not occur within these specified times, the unfertilized oocytes will undergo an aging process and lose their ability to be fertilized. There have been no data, however, on

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Table 2 Effect of trichostatin A (TSA) supplementation during extended culture for 6 h on fertilization and subsequent development

			After extended culture						
Treatment group	GV	MII	Intact	Spontaneously activated	Fragmented	Lysed	Inseminated	2-cells (%)	Blastocyst (%)
TSA 0 nM	53	32	32	–	–	–	32	22 (68.8)	1 (4.5)
TSA 100 nM	81	60	60	–	–	–	60	43 (71.7)	1 (2.3)
TSA 500 nM	79	57	57	–	–	–	57	43 (75.4)	2 (4.7)

Table 3 Effect of trichostatin A (TSA) supplementation during extended culture for 12 h on fertilization and subsequent development

			After extended culture						
Treatment group	GV	MII	Intact	Spontaneously activated	Fragmented	Lysed	Inseminated	2-cells (%)	Blastocyst
TSA 0 nM	27	12	12	0	0	0	12	4 (33.3)	0
TSA 100 nM	40	30	30	0	0	0	30	12 (40.0)	0
TSA 500 nM	45	30	30	0	0	0	30	14 (46.7)	0

Table 4 Effect of trichostatin A (TSA) supplementation during extended culture for 24 h on parthenogenetic activation and subsequent development

			After extended culture						
Treatment group	GV	MII	Intact	Spontaneously activated	Fragmented	Lysed	Artificially activated[a] (%)	2-cells (%)	Blastocyst
TSA 0 nM	41	25	25	0	0	0	25 (100)	8 (32.0)	0
TSA 100 nM	81	45	45	0	0	0	45 (100)	6 (13.3)	0
TSA 500 nM	89	53	53	0	0	0	53 (100)	14 (26.4)	0

[a]Artificial parthenogenetic activation was induced by 10 mM $SrCl_2$ for 2.5 h.

the optimal fertilization period for *in vitro* matured oocytes. In the present study, we collected immature mouse oocytes and induced *in vitro* maturation. After *in vitro* maturation, the matured oocytes were further cultured *in vitro* to induce the aging process, and the ability of fertilization and blastocyst formation was evaluated. After 12 h of culture, no blastocysts had developed; thus, at least 12 hour's *in vitro* aging is an inadequate amount of time for normal embryo development. If fertilization does not occur, the unfertilized mature oocytes remaining *in vivo* or *in vitro* will undergo an aging process. This aging process is also observed in oocytes from aged ovaries; ovarian function is gradually decreased in aged animals, and even in MII oocytes its ability is significantly decreased (Tatone *et al.*, 2008).

In the present study, we analysed the effect of histone acetylation induced by the supplementation of TSA on fertilization and embryo development during the *in vitro* aging of *in vitro* matured mouse oocytes. TSA supplementation did not improve fertilization and blastocyst formation after 6 and 12 h of extended culture. A previous study with porcine oocytes showed that supplementation of 100 nM of TSA significantly increased the blastocyst formation rate. In the same study, the fertilization rate was not significantly different (Jeseta *et al.*, 2008). The difference in species could explain the different results of the two studies. However, the previous study, which showed the beneficial effect of TSA, could not explain the significant decrease of the blastocyst formation rate in the group that had a higher TSA dose supplementation (1000 nM).

A previous study demonstrated that histone acetylation increased as oocyte aging progressed (Huang *et al.*, 2007). Thus, the assumption that the supplementation of TSA, which induces histone acetylation, might be beneficial to embryo development could be controversial. In that study, however, the histone acetylation of mouse oocytes was increased on H4K8 and H4K12 after 5 h of *in vitro* aging and on H3K14, H4K8, and H4K12 after 10 h of *in vitro* aging. These results indicate that not every lysine on H3 and H4 is acetylated as part of the aging progress. Moreover,

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in vivo matured oocytes were used in that study, which is different from the *in vitro* matured oocytes we used in the present study.

Reports on the effect of TSA supplementation during the *in vitro* maturation of immature oocytes are not consistent. Several studies have shown that there was no difference in the *in vitro* maturation rate in murine and porcine oocytes according to HDAC inhibition (Kim *et al.*, 2003; Endo *et al.*, 2005). However other investigators reported maturation delay in porcine oocytes (Wang *et al.*, 2006; Bui *et al.*, 2007; Jeseta *et al.*, 2008; Petr *et al.*, 2009). Considering these controversial results, further investigation is necessary to elucidate the association of histone acetylation with the various physiologic and pathologic phenomena of oocytes.

In the present study, 5/80 spontaneously activated and 11/80 fragmented oocytes were observed after 24 h of extended *in vitro* culture in Experiment 1. In Experiment 3, however, no oocytes underwent activation, fragmentation, or lysis. The small number of oocytes analysed in Experiment 3 could be the cause of this difference. In Experiment 3, parthenogenesis was induced in all oocytes after 24 h of extended culture, regardless of TSA supplementation. It is well known that the aged oocyte has an increased sensitivity to external stimuli (Goud *et al.*, 1999). In the present study, fewer oocytes progressed to the 2-cell stage after artificial parthenogenesis in the TSA supplementation groups than in the control group. This finding suggests that TSA supplementation is not beneficial for the development of parthenotes.

Investigations of the various acetyl expression patterns of histones H3 and H4 as well as the spindle and chromosomal statuses are necessary to elucidate the effect of HDAC inhibition on oocyte development and aging. In addition, an analysis and comparison of the various effects of other HDAC inhibitors, such as valproic acid and sodium butyrate, as well as TSA may also be necessary for further understanding of this issue (Schwartz *et al.*, 1998; Endo *et al.*, 2008).

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