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Protective effects of ethanol extracts of *Artemisia asiatica* Nakai ex Pamp. on ageing-induced deterioration in mouse oocyte quality

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Summary

Following ovulation, oocytes undergo a time-dependent deterioration in quality referred to as post-ovulatory ageing. Although various factors influence the post-ovulatory ageing of oocytes, oxidative stress is a key factor involved in deterioration of oocyte quality. *Artemisia asiatica* Nakai ex Pamp. has been widely used in East Asia as a food ingredient and traditional medicine for the treatment of inflammation, cancer, and microbial infections. Recent studies have shown that *A. asiatica* exhibits antioxidative effects. In this study, we investigated whether *A. asiatica* has the potential to attenuate deterioration in oocyte quality during post-ovulatory ageing. Freshly ovulated mouse oocytes were cultured with 0, 50, 100 or 200 μg/ml ethanol extracts of *A. asiatica* Nakai ex Pamp. After culture for up to 24 h, various ageing-induced oocyte abnormalities, including morphological changes, reactive oxygen species (ROS) accumulation, apoptosis, chromosome and spindle defects, and mitochondrial aggregation were determined. Treatment of oocytes with *A. asiatica* extracts reduced ageing-induced morphological changes. Moreover, *A. asiatica* extracts decreased ROS generation and the onset of apoptosis by preventing elevation of the Bax/Bcl-2 expression ratio during post-ovulatory ageing. Furthermore, *A. asiatica* extracts attenuated the ageing-induced abnormalities including spindle defects, chromosome misalignment and mitochondrial aggregation. Our results demonstrate that *A. asiatica* can relieve deterioration in oocyte quality and delay the onset of apoptosis during post-ovulatory ageing.

Keywords: Apoptosis, *Artemisia asiatica*, Oocytes, Post-ovulatory ageing, Reactive oxygen species

Introduction

Oocyte quality has been defined as its abilities to be fertilized, mature, and give rise to normal offspring (Duranthon & Renard, 2001). Good quality of oocytes is essential for fertilization and subsequent embryonic development. However, if fertilization does not occur for a prolonged period of time after ovulation, oocytes progressively undergo a time-dependent deterioration in quality, referred to as post-ovulatory ageing (Miao *et al*., 2009). Post-ovulatory ageing displays a range of morphological, cellular and molecular changes, including spontaneous activation, partial cortical granule exocytosis, zona pellucida hardening, mitochondrial dysfunction, spindle abnormalities and loss of chromosome integrity (Dodson *et al*., 1989; Ducibella *et al*., 1990; Xu *et al*., 1997; Longo, 1981; Wakayama *et al*., 2004; Takahashi *et al*., 2003; Tatone *et al*., 2011; Zhang *et al*., 2011; Premkumar & Chaube, 2013). These abnormalities may affect the developmental competence and fertilization rates, increasing abnormal and/or retarded development of embryo/fetuses (Szollosi, 1971; Winston *et al*., 1993; Tarin *et al*., 1999; Fissore *et al*., 2002; Takahashi *et al*., 2003). Moreover, severe deterioration in oocyte quality ultimately leads to apoptosis (Fujino *et al*., 1996). Although various factors influence the post-ovulatory ageing of oocytes, oxidative stress is considered to

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be one of the key factors involved in deterioration of oocyte quality (Lord & Aitken, 2013). Indeed, ROS levels have been found to increase with oocyte age and oxidative stress-induced apoptosis during *in vitro* culture of oocytes (Takahashi *et al*., 2003; Chaube *et al*., 2005). Consistent with this, it has been shown that the addition of antioxidants, such as glutathione, vitamin E, and vitamin C, to the culture medium improves the rate of *in vitro* maturation and subsequent embryonic development (Eppig *et al*., 2000; de Matos & Furnus, 2000; Tao *et al*., 2004; Luberda, 2005; Choi *et al*., 2007). Therefore, it is of interest to develop methods that protect oocytes from oxidative stress to preserve oocyte quality.

Artemisia asiatica Nakai ex Pamp. has been widely used in East Asia to treat inflammation, cancer, and microbial infections (Huh *et al*., 2003; Song *et al*., 2008). Recent studies have shown that *A. asiatica* exhibits antioxidative and anti-inflammatory effects, which contribute to its protective effects against various pathophysiological conditions such as gastric damage, liver damage, pancreatitis, and tumour promotion (Hahm *et al*., 1998; Oh *et al*., 2005; Ryu *et al*., 1998; Seo *et al*., 2002). Currently, a formulated ethanol extract of *A. asiatica*, DA-9601 (Stillen^{TM}) is clinically used to treat gastric mucosal ulcers and inflammation (Seol *et al*., 2004). Moreover, eupatilin, an active compound isolated from *A. asiatica*, has been reported to have antioxidant and anti-inflammatory effects against gastric mucosal injury (Oh *et al*., 2001).

Given that ROS is a primary factor involved in deterioration of oocyte quality and that *A. asiatica* has antioxidant activity, it is possible to hypothesize that *A. asiatica* may relieve post-ovulatory ageing of oocytes by reducing oxidative stress during *in vitro* culture of oocytes. Therefore, in the present study we investigated the effects of *A. asiatica* on post-ovulatory ageing of oocytes. We found that *A. asiatica* could effectively reduce the ROS level in oocytes during *in vitro* culture, delaying deterioration in quality and the onset of apoptosis. Therefore, our results provide the first evidence that *A. asiatica* may have beneficial effects on oocyte quality.

Materials and methods

Materials

A 95% ethanol extract (Code No: CA02-070) of *A. asiatica* Nakai ex Pamp. was purchased from the Plant Extract Bank of the Plant Diversity Research Center (Daejeon, Korea). A stock solution (350 mg/ml) of *A. asiatica* ethanol extract was prepared in 100% dimethyl sulfoxide (DMSO) and diluted to 0–200 μg/ml with M16 medium for *in vitro* culture. All other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated.

Animals

Female CD-1 mice (3- to 4-week-old) were purchased from Koatech (Pyeongtaek, Korea). All procedures for mouse care and use were conducted in accordance with the guidelines and approved by the Institutional Animal Care and Use Committees of Sungkyunkwan University (approval ID:SKKU 12–37).

Oocyte collection and culture

Mice were superovulated by injection of pregnant mare's serum of gonadotrophin (PMSG, 5 IU) followed by human chorionic gonadotrophin (hCG, 5 IU) 46–48 h later. Oocytes were collected in M2 medium at 13–14 h after hCG injection, and cumulus cells were removed using hyaluronidase. After washing, cumulus-free oocytes were cultured in M16 medium in a 5% CO_2 in air atmosphere at 37°C with or without ethanol extract of *A. asiatica*.

Immunoblotting analysis

Oocytes were lysed in SDS sample buffer and subjected to SDS-PAGE. After transfer, the membranes were blocked in TBST (0.1% Tween-20, 3% BSA) at room temperature for 1 h, and then incubated with primary antibodies overnight at 4°C. After washing three times in TBST, membranes were incubated with secondary antibodies for 1 h. The blots were developed with the ECL Plus Western Blotting Detection kit (GE Healthcare Life Science, Marlborough, MA, USA). The primary antibodies were anti-Bax (Cell Signaling, Beverly, MA, USA, 1:1000), anti-Bcl-2 (Cell Signaling, 1:1000) and anti-β actin (Cell Signaling, 1:500). The HRP-labelled mouse and rabbit antibodies (Jackson ImmunoResearch, Westgrove, PA, USA) were used as the secondary antibodies for immunoblotting.

Measurement of intracellular ROS level

Intracellular ROS levels were detected using dihydrorhodamine 123 (DHR-123). Briefly, oocytes were incubated with 20 μM DHR-123 for 30 min before visualization under fluorescence microscopy. The fluorescence signals were acquired using a Nikon Eclipse Ti inverted microscope with a CCD-cooled camera (DS-Qi1Mc, Nikon, Tokyo, Japan) and quantified using ImageJ software (National Institutes of Health, MD, USA).

Annexin V staining

Oocytes were incubated with FITC annexin V (BD Pharmingen, San Diego, CA, USA) for 15 min and

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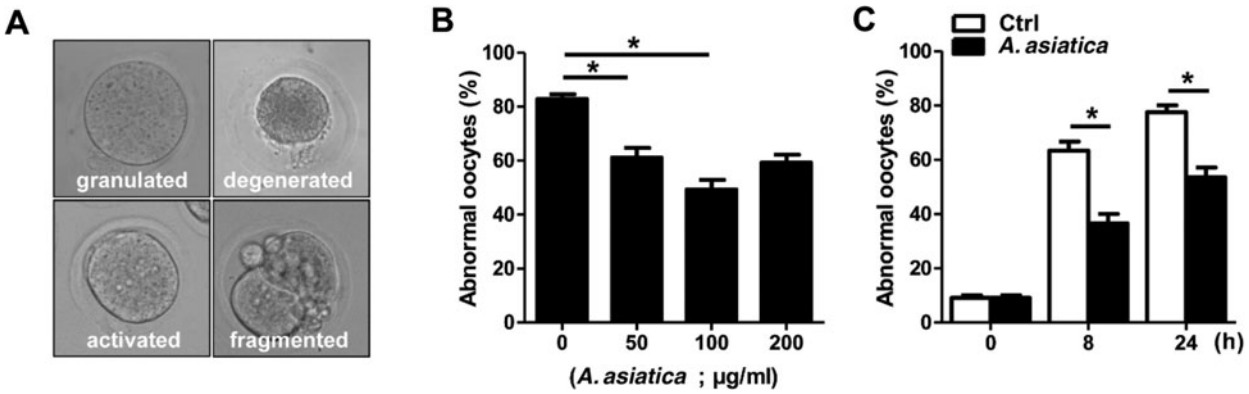

Figure 1 Effects of *Artemisia asiatica* on oocyte morphology during post-ovulatory ageing. (*A*, *B*) Oocytes were cultured for 12 h with 0, 50, 100 or 200 μg/ml of *Artemisia asiatica* ethanol extract. (*A*) Representative abnormal morphologies of oocytes after *in vitro* culture are shown. (*B*) Oocytes with abnormal morphologies were quantified. (*C*) Oocytes were cultured for up to 24 h with 100 μg/ml *A. asiatica* ethanol extract. Oocytes with abnormal morphologies were quantified. $^{*}P < 0.05$.

washed three times in M2 medium. Fluorescent signals were measured using a Nikon Eclipse Ti inverted microscope with a CCD-cooled-camera (DS-Qi1Mc, Nikon) and quantified using ImageJ software (National Institutes of Health).

Immunostaining and confocal microscopy

Oocytes were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min, permeabilized in PBS with 0.1% Triton X-100 for 15 min, and blocked in PBS containing 3% BSA for 1 h. Immunostaining was performed using primary antibodies against acetylated α-tubulin (1:500), followed by Alex Fluor-conjugated 488 secondary antibodies (Jackson ImmunoResearch). DNA was counterstained with DAPI. For mitochondrial staining, oocytes were stained with CytoPainter MitoRed (Abcam, Cambridge, UK) for 30 min. After washing, the oocytes were mounted on glass slides and examined with a confocal laser-scanning microscopy (LSM 700; Zeiss, Berlin, Germany) equipped with a C-Apochromat ×63/1.2 water immersion objective. Data analysis was performed using the ZEN 2010 LSM software (Zeiss) and ImageJ software (National Institutes of Health).

Statistical analysis

Statistical analysis was performed with GraphPad Prism (GraphPad Software, San Diego, CA, USA). Data are representative of at least three independent experiments unless otherwise specified and each experimental group included at least 20 oocytes. The significance of differences between groups was analysed by Student's *t*-test; *P*-values < 0.05 were considered to be statistically significant.

Results

Artemisia asiatica reduces morphological changes during post-ovulatory oocyte ageing

To investigate the effect of *A. asiatica* on ageing-induced morphological changes, we treated oocytes with ethanol extracts of *A. asiatica* during *in vitro* culture. After 12 h of culture, oocytes showed various morphological defects, including granulation, fragmentation, parthenogenetic activation and degeneration (Fig. 1*A*). While untreated oocytes exhibited various abnormal morphologies, oocytes treated with *A. asiatica* showed a significant decrease in abnormal morphologies in a dose-dependent manner (Fig. 1*B*). However, the preventive effects of *A. asiatica* on ageing-induced morphological changes were not further increased at 200 μg/ml of *A. asiatica*, suggesting that 100 μg/ml of *A. asiatica* is the optimal concentration to reduce morphological changes during post-ovulatory oocyte ageing.

We next determined the time-dependent effect of *A. asiatica* on ageing-induced abnormal morphologies. Oocytes were cultured for up to 24 h with or without 100 μg/ml of *A. asiatica* and morphological changes in oocytes were assessed. After 8 h of culture with *A. asiatica*, oocytes with abnormal morphologies were significantly reduced. Similarly, we also observed reduced morphological defects after 24 h of culture (Fig. 1*C*). These results suggest that *A. asiatica* reduces morphological changes during post-ovulatory ageing of oocytes.

Artemisia asiatica decreases ROS accumulation in ageing oocytes

To further examine the effects of *A. asiatica* on oocyte quality, we investigated intracellular ROS generation

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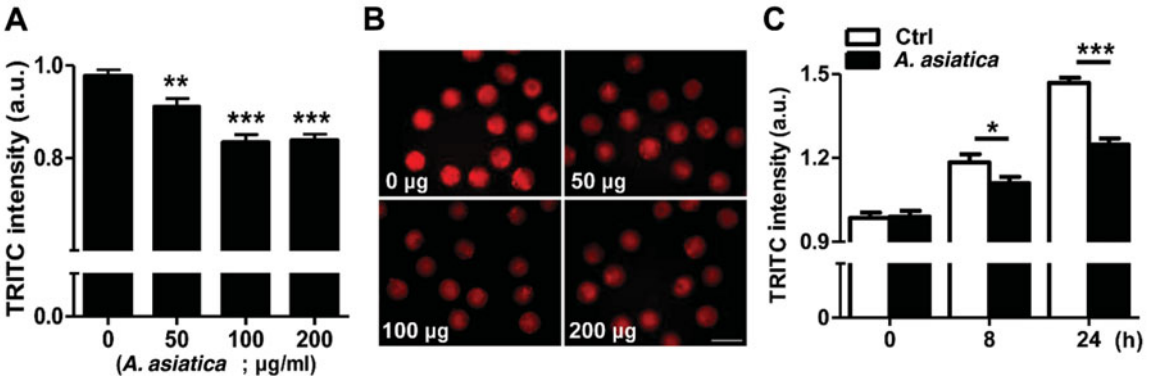

Figure 2 Effects of *Artemisia asiatica* on ROS generation in ageing oocytes. (*A*, *B*) Oocytes were cultured for 12 h with 0, 50, 100 or 200 μg/ml of *Artemisia asiatica* ethanol extract. (*A*) The fluorescence intensity was measured following incubation with DHR-123 for 30 min. (*B*) Representative images are shown. Bar, 100 μm. (*C*) Oocytes were cultured for up to 24 h with 100 μg/ml of *A. asiatica* ethanol extract. The fluorescence intensity was measured following incubation with DHR-123 for 30 min. $^{*}P < 0.05$, $^{**}P < 0.001$, $^{***}P < 0.0001$.

during post-ovulatory ageing of oocytes. After 12 h of *in vitro* culture, we observed that *A. asiatica* treatment resulted in a significant decrease in ROS accumulation in a dose-dependent manner (Fig. 2*A*, *B*). We next assessed the time-dependent effects of *A. asiatica* on ROS accumulation in ageing oocytes. Similarly, the elevation of ROS level was retarded in oocytes treated with *A. asiatica* (Fig. 2*C*).

Artemisia asiatica delays the onset of apoptosis during post-ovulatory ageing of oocytes

Because oxidative stress has been shown to accelerate the onset of apoptosis in oocytes, we examined whether *A. asiatica* reduced the onset of apoptosis. Similar to levels of ROS, the levels of annexin V, a hallmark of the early phase of apoptosis (Martin *et al.*, 1995), were decreased in a dose-dependent manner following *A. asiatica* treatment for 12 h (Fig. 3*A*, *B*). We also observed that *A. asiatica* significantly delayed the increase in annexin V intensity during *in vitro* culture of oocytes (Fig. 3*C*). In conjunction with reduced ROS levels in oocyte culture with *A. asiatica*, this result demonstrates that *A. asiatica* delays the onset of apoptosis during post-ovulatory oocyte ageing.

We next determined the levels of Bax and Bcl-2 following *A. asiatica* treatment. Immunoblot analysis revealed that Bax expression increased, whereas Bcl-2 expression decreased after *in vitro* culture of oocytes. Thus, the ratio of Bax/Bcl-2 was significantly increased during post-ovulatory ageing of oocytes. However, *A. asiatica* reduced upregulation of Bax and downregulation of Bcl-2 during *in vitro* culture of oocytes, thereby reducing the elevation of the Bax/Bcl-2 ratio (Fig. 4*A*–*C*). These results suggest that *A. asiatica* delays the onset of apoptosis during post-ovulatory ageing of oocytes.

Artemisia asiatica attenuates ageing-induced spindle and chromosome abnormalities

We next determined the effect of *A. asiatica* on the chromosome alignment and spindle organization, which are the most critical events involved in ensuring developmental competency of the oocytes. Whereas fresh oocytes displayed a typical barrel-shaped spindle, spindles became elongated and less integrated in aged oocytes. Furthermore, aberrant chromosome alignment was increased in aged oocytes. However, these abnormalities in spindle organization and chromosome alignment were significantly reduced after *A. asiatica* treatment (Fig. 5*A*, *B*).

Artemisia asiatica reduces mitochondrial aggregation during post-ovulatory ageing

Because mitochondria distribution is correlated with oocyte quality and developmental competency in aged oocytes, we examined whether mitochondrial distribution was affected by *A. asiatica* treatment during post-ovulatory ageing of oocytes (Nagai *et al.*, 2006). Confocal analysis with mitochondria probe revealed that mitochondria were evenly distributed in the cytoplasm in fresh oocytes, but extensively aggregated after 12 h of *in vitro* culture. However, these aggregations were significantly reduced in oocytes treated with *A. asiatica* (Fig. 6*A*, *B*). Taken together, our data demonstrate that *A. asiatica* prevents the deterioration of oocyte quality during post-ovulatory ageing of oocytes.

Discussion

The effect of *A. asiatica* on oocyte quality, especially during post-ovulatory ageing, has not been

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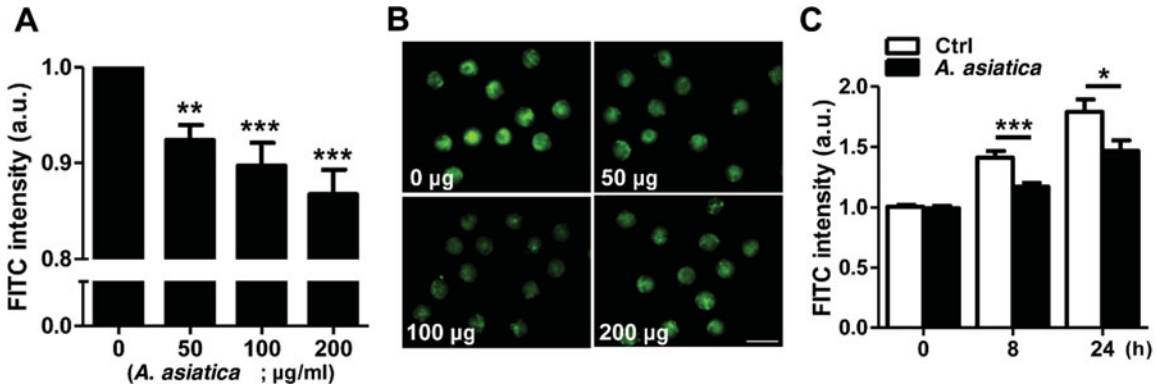

Figure 3 Effects of *Artemisia asiatica* on oocyte apoptosis during post-ovulatory ageing. (*A*, *B*) Oocytes were cultured for 12 h with 0, 50, 100 or 200 μg/ml of *Artemisia asiatica* ethanol extract. (*A*) The fluorescence intensity was measured following incubation with annexin V for 15 min. (*B*) Representative images are shown. Bar, 100 μm. (*C*) Oocytes were cultured for up to 24 h with 100 μg/ml of *A. asiatica* ethanol extract. The fluorescence intensity was measured following incubation with annexin V for 15 min. $^{*}P < 0.05$, $^{**}P < 0.001$, $^{***}P < 0.0001$.

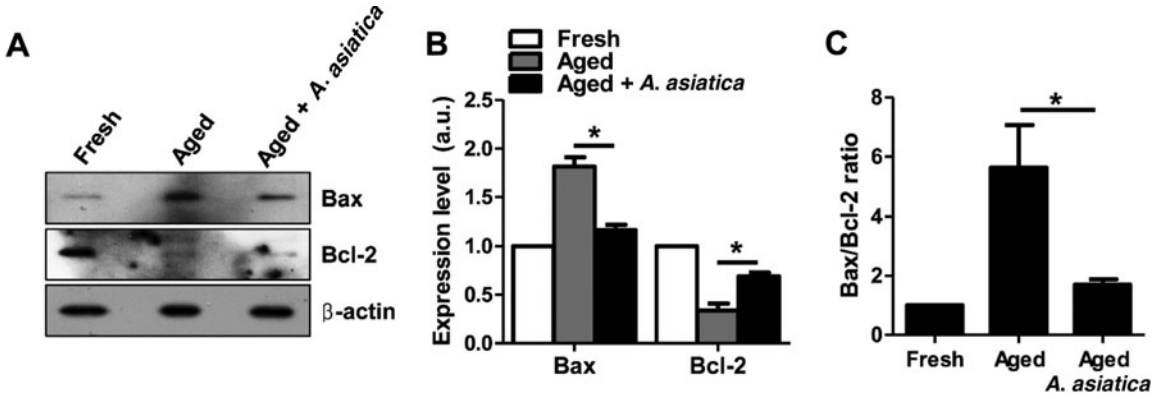

Figure 4 Effects of *Artemisia asiatica* on the expression of anti- and pro-apoptotic proteins during post-ovulatory ageing. Oocytes were cultured for 12 h with or without 100 μg/ml of *Artemisia asiatica* ethanol extract. (*A*) The expression levels of Bax and Bcl-2 were determined by immunoblot analyses. β-Actin was used as a loading control. (*B*) The normalized levels of Bax and Bcl-2 are shown. (*C*) The relative Bax/Bcl-2 expression ratio is shown. $^{*}P < 0.05$.

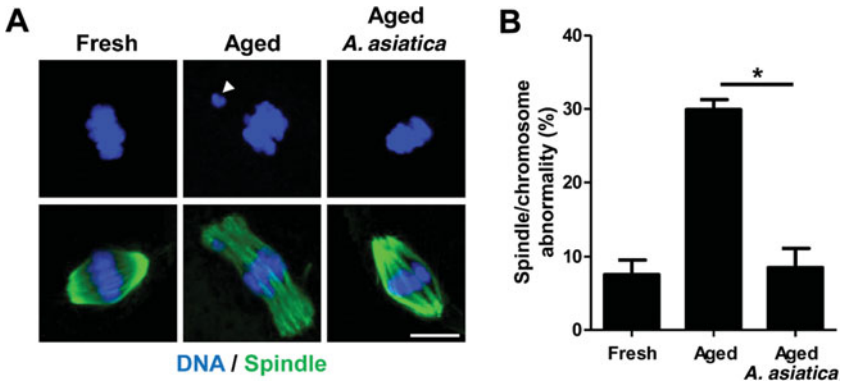

Figure 5 Effects of *Artemisia asiatica* on spindle and chromosome misalignment defects during post-ovulatory ageing. Oocytes were cultured for 12 h with or without 100 μg/ml of *Artemisia asiatica* ethanol extract. (*A*) Spindle and chromosome abnormalities were determined by immunostaining with anti-tubulin antibody and DAPI. The representative images are shown from three independent experiments. Arrowhead indicates the misaligned chromosomes. Bar, 10 μm. (*B*) The incidence of spindle and chromosome abnormality is shown. $^{*}P < 0.05$.

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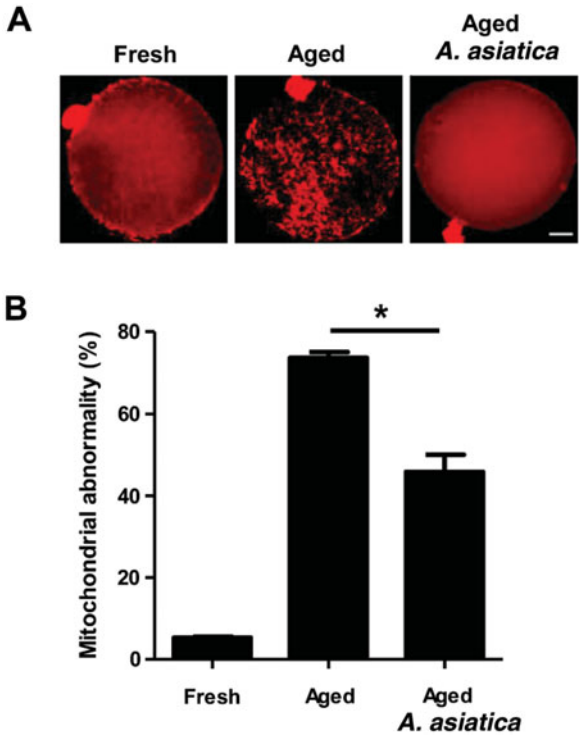

Figure 6 Effects of *Artemisia asiatica* on mitochondrial distribution during post-ovulatory ageing. Oocytes were cultured for 12 h with or without 100 μg/ml of *Artemisia asiatica* ethanol extract. (*A*) Mitochondrial distribution was determined by immunostaining with CytoPainter MitoRed. Bar, 10 μm. (*B*) The incidence of mitochondrial aggregation is shown. $^{*}P < 0.05$.

investigated. In this study, we demonstrated that *A. asiatica* has the potential to attenuate the deterioration in oocyte quality during post-ovulatory ageing.

Post-ovulatory ageing has been shown to induce many morphological changes, such as increased cytoplasmic granules, fragmentation and spontaneous activation. Moreover, the increase in incidence of oocytes exhibiting spindle defects, chromosome misalignment and mitochondrial aggregation is a hallmark of ageing-related decline in oocyte quality (Lord and Aitken, 2013). *Artemisia asiatica* has been shown to have various beneficial effects on health by exhibiting anti-inflammatory and antioxidant activities as well as anti-cancer effects (Hahm *et al*., 1998; Oh *et al*., 2005; Ryu *et al*., 1998; Seo *et al*., 2002). However, the effects of *A. asiatica* on reproductive health have not been reported. Our results showed that morphological and cellular abnormalities were significantly decreased in oocytes treated with *A. asiatica*. These results suggest that *A. asiatica* also has beneficial effects on reproductive health.

To further examine the effects of *A. asiatica* on oocyte quality during post-ovulatory ageing, we measured the levels of ROS in ageing oocytes. It was reported that ROS accumulate during *in vitro* culture of oocytes and are considered to be a key factor that influences the oocyte quality (Takahashi *et al*., 2003; Lord & Aitken, 2013). Consistent with this, we observed that the extended culture of oocytes *in vitro* results in increased ROS levels. However, the accumulation of ROS was significantly reduced when oocytes were treated with *A. asiatica*, implying that *A. asiatica* has antioxidant effects. Indeed, it has been well documented that *A. asiatica* has antioxidant effects (Hahm *et al*., 1998; Ryu *et al*., 1998; Seo *et al*., 2002; Oh *et al*., 2005).

Overproduction of ROS may cause oxidative stress and ultimately result in apoptosis in oocytes (Agarwal *et al*., 2008). To investigate the effects of *A. asiatica* on apoptosis associated with oocyte ageing, we used annexin V, a phospholipid-binding protein that detects the onset of apoptosis (Martin *et al*., 1995). Our results suggest that *A. asiatica* delays the onset of apoptosis, showing reduced levels of annexin V. To further investigate the effects of *A. asiatica* on ageing-induced apoptosis, we examined the level of apoptosis-related proteins, Bax and Bcl-2. It has been reported that Bcl-2 expression decreases, whereas Bax expression increases in the oocytes aged *in vitro* (Perez *et al*., 2005; Steuerwald *et al*., 2005). Consistent with this, we observed a decrease in Bcl-2 and increase in Bax expression, thereby elevating the Bax/Bcl-2 ratio during *in vitro* culture of oocytes. However, after *A. asiatica* treatment, the expression ratio of Bax/Bcl-2 was not elevated during post-ovulatory ageing of oocytes. Therefore, our data suggest that *A. asiatica* delays the onset of apoptosis during *in vitro* culture of oocytes by preventing elevation of the Bax/Bcl-2 ratio.

Assisted reproductive technology (ART) refers to all procedures that involve handling oocytes or embryos *in vitro* to achieve successful pregnancy. In humans, the post-ovulatory ageing of oocytes is one of the causes of failure in ART. Therefore, the establishment of methods to prevent oocyte ageing might enhance progress in ART procedures. In this study, we have found that supplementation with *A. asiatica* in the culture medium relieves multiple facets of oocyte ageing and apoptosis *in vitro*, thereby preventing the age-associated decline in oocyte quality. Therefore, our data provide the first evidence that *A. asiatica* may be a promising treatment for oocyte ageing during prolonged culture times, which is particularly important for ART procedures.

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Conflict of interest

The authors declare no competing financial interests.

Authors' contributions

J.O. conceived and designed the experiments. H.J., S.Y. and D.K. performed the experiments. J.O. analysed the data. H.J. contributed reagents and materials. J.O. wrote the paper. All authors reviewed the manuscript.

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