Effects of heat stress during in vitro maturation on cytoplasmic versus nuclear components of mouse oocytes

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Abstract

The objectives of this study were to investigate the effect of heat stress during in vitro maturation on the developmental potential of mouse oocytes and to determine whether the deleterious effect was on the nuclear or cytoplasmic component. While rates of oocyte nuclear maturation (development to the metaphase II stage) did not differ from 37 to 40 °C, rates for blastocyst formation decreased significantly as maturation temperature increased from 38.5 to 39 °C. Chromosome spindle exchange showed that while blastocyst formation did not differ when spindles matured in vivo or in vitro at 37, 40 or 40.7 °C were transplanted into in vivo matured cytoplasts, no blastocyst formation was observed when in vivo spindles were transferred into the 40 °C cytoplasts. While oocytes reconstructed between 37 °C ooplasts and 37 or 40 °C karyoplasts developed into 4-cell embryos at a similar rate, no oocytes reconstituted between 40 °C ooplasts and 37 °C spindles developed to the 4-cell stage. Immunofluorescence microscopy revealed impaired migration of cortical granules and mitochondria in oocytes matured at 40 °C compared with oocytes matured at 37 °C. A decreased glutathione/GSSG ratio was also observed in oocytes matured at 40 °C. While spindle assembling was normal and no MAD2 was activated in oocytes matured at 37 or 40 °C, spindle assembling was affected and MAD2 was activated in some of the oocytes matured at 40.7 °C. It is concluded that 1) oocyte cytoplasmic maturation is more susceptible to heat stress than nuclear maturation, and 2) cytoplasmic rather than nuclear components determine the pre-implantation developmental capacity of an oocyte.

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Introduction

Early studies showed that farm animals such as cattle (Badinga et al. 1985, Cavestany et al. 1985), pigs (Omtvedt et al. 1971), and sheep (Dutt 1963) had lower fertility in the summer than in any other season. Recent studies demonstrated that heat stress caused infertility not only by affecting hormonal secretion (Wolfenson et al. 2000) and embryo development (Edwards & Hansen 1997, Rivera & Hansen 2001) but also by damaging the oocyte. For example, oocytes harvested from cows during the summer showed reduced ability to develop into blastocysts after fertilization in vitro (Rocha et al. 1998, Rutledge et al. 1999, Al-Katanani et al. 2002). Exposure of heifers to heat stress between the onset of estrus and insemination increased the proportion of abnormal and retarded embryos (Putney et al. 1989). This suggests that the process of oocyte maturation is susceptible to heat stress. In fact, it has been shown that exposure of bovine oocytes to elevated temperature during in vitro maturation decreased their subsequent cleavage and blastocyst rates (Edwards et al. 1997, Roth & Hansen 2004).

Oocyte maturation involves both nuclear and cytoplasmic events. While nuclear maturation includes the resumption of the first meiotic division and the progression of meiosis to the metaphase II stage, cytoplasmic maturation includes a series of processes that are necessary for the oocyte to acquire the capacity to support male pronucleus formation, monospermic fertilization, and early embryonic development (Eppig et al. 1996). The completion of nuclear maturation in vitro does not assure the completion of normal cytoplasmic maturation. Thus, although spontaneous nuclear maturation of oocytes appears to occur normally in vitro, the functionality of the oocytes for post-fertilization development is apparently not completely normal because development of the in vitro matured bovine oocytes to the preimplantation stages following IVF is less successful than that of oocytes matured in vivo (Leibfried-Rutledge et al. 1987, Eppig 1996, Van de Leemput et al. 1999). Chromosomal spindle exchange experiments between metaphase II oocytes showed that in vitro maturation (Liu et al. 2003a) and post-ovulatory aging processes (Bai et al. 2006) reduced the developmental potential of mouse oocytes by affecting the cytoplasmic components rather than the nuclear components. However, whether heat stress during oocyte maturation affects the nuclear or cytoplasmic
component is unclear. In addition, reports on the effect of hyperthermia on the maturing mouse oocytes are few (Fiorensa & Mangia 1992), and the effect of heat stress during *in vitro* maturation on the developmental potential of mouse oocytes has not been reported, to our knowledge.

The objectives of the present study were to investigate the effect of heat stress during *in vitro* maturation on the developmental potential of mouse oocytes and to determine whether the detrimental effect is on the nuclear or cytoplasmic component. The critical hyperthermal temperature for *in vitro* maturation of mouse oocytes was first determined by observing nuclear maturation to metaphase II and preimplantation development after oocytes were matured *in vitro* under different culture temperatures. The effect of heat stress on oocyte nuclear or cytoplasmic component was then differentiated by observing development following chromosome spindle exchange between the heat stressed and unstressed metaphase II oocytes. Finally, cytoplasmic changes were examined after oocytes were matured under heat stress. The results indicated that 1) oocyte cytoplasmic maturation is more susceptible to heat stress than nuclear maturation to metaphase II and 2) cytoplasmic rather than nuclear components determine the pre-implantation developmental capacity of an oocyte.

**Results**

**Maturation and embryo development of mouse oocytes matured at different temperatures**

Freshly collected germinal vesicle stage oocytes were cultured at different temperatures for 14 h for maturation. At the end of maturation culture, the oocytes were aged for 10 h at 37 °C. Then, the oocytes were treated for activation and activated oocytes were cultured for embryo development. While rates of oocyte maturation to metaphase II did not differ from 37 to 40 °C, rates for blastocyst formation decreased significantly as maturation temperature increased from 38.5 to 39 °C (Table 1). Only 30% of the oocytes matured after culture at 40.7 °C and those matured lost the ability to activate. No oocytes matured to metaphase II following culture at 41 °C.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Percentage of oocytes matured</th>
<th>Percentage of 2-cell</th>
<th>Percentage of 4-cell</th>
<th>Percentage of blastocysts</th>
</tr>
</thead>
<tbody>
<tr>
<td>37</td>
<td>98.9 ± 1.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>89.9 ± 3.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>75.0 ± 7.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>27.2 ± 2.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>38.5</td>
<td>98.7 ± 1.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>87.4 ± 4.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>61.1 ± 3.4&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>25.4 ± 1.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>39</td>
<td>99.0 ± 1.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>88.0 ± 4.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>46.5 ± 8.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.0 ± 1.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>39.5</td>
<td>99.1 ± 1.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>79.7 ± 3.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25.3 ± 5.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5.5 ± 0.6&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>40</td>
<td>98.9 ± 1.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>47.3 ± 3.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.0 ± 0.0&lt;sup&gt;d&lt;/sup&gt;</td>
<td>–</td>
</tr>
<tr>
<td>40.7</td>
<td>28.3 ± 1.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>50.3 ± 1.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.0 ± 0.0&lt;sup&gt;d&lt;/sup&gt;</td>
<td>–</td>
</tr>
<tr>
<td>41</td>
<td>0.9 ± 0.0&lt;sup&gt;h&lt;/sup&gt;</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

<sup>a–d</sup>Values with a common letter in their superscripts did not differ (*P > 0.05*). Each treatment was repeated 3–4 times and each replicate consisted of 25–30 oocytes.

**Embryonic development after chromosomal spindle exchange between mouse oocytes matured in vivo or in vitro at different temperatures**

Chromosomal spindles were exchanged between mouse oocytes matured *in vivo* or *in vitro* at different temperatures. The reconstructed oocytes were activated with SrCl2 for embryo development. When spindles from oocytes matured at 37 or 40 °C were transferred into cytoplasts matured at 37 °C, the reconstructed oocytes developed into 4-cell embryos at a similar rate (Table 2). When spindles matured at 37 °C were transferred into cytoplasts matured at 40 °C, however, no reconstructed oocytes developed to the 4-cell stage. When spindles matured at 37, 40 or 40.7 °C were placed into the *in vivo* matured cytoplasts, percentages of blastocyst formation and cell number per blastocyst did not differ between different treatments and were similar to those of the control oocytes reconstituted with spindles and cytoplasts both matured *in vivo*. However, when the *in vivo* spindles were transplanted into *in vitro* ooplasm, the embryo development rates decreased significantly. Furthermore, rates of 4-cell embryos were significantly lower and no blastocyst formation was observed in oocytes reconstituted with ooplasm matured at 40 °C compared with oocytes reconstituted with the 37 °C ooplasm. In addition, the *in vivo* karyoplasts supported a better embryo development than karyoplasts matured *in vitro* at 37 °C when combined with cytoplasts matured *in vitro* at 37 or 40 °C. The results indicated that 1) heat stress during maturation affected mainly the cytoplasm rather than the nuclear components of mouse oocytes; 2) it was the cytoplasm but not the karyoplast that determined the pre-implantation developmental potential of reconstructed oocytes; and 3) oocyte cytoplasmic maturation was better accomplished *in vivo* than *in vitro*.

**Effects of heat stress on the distribution of cortical granules and mitochondria in mouse oocytes**

The distribution of cortical granules and mitochondria was observed in mouse oocytes matured under different temperatures for 14 h. When observed under a laser confocal microscope following staining, oocytes were classified as those with complete or incomplete migration...
of cortical granules, and those with complete or incomplete congregation of mitochondria. While almost all the cortical granules were located in the egg cortex with few or no in the inner cytoplasm in oocytes with complete migration of cortical granules (Fig. 1A), only some cortical granules migrated to the cortex with many left in the inner ooplasm in oocytes with incomplete migration of cortical granules (Fig. 1B). While most of the mitochondria aggregated in the animal hemisphere around the spindle in oocytes with complete congregation of mitochondria (Fig. 1D), only some mitochondria aggregated in the animal hemisphere with many left in the vegetal hemisphere in oocytes with incomplete mitochondrial congregation (Fig. 1C). Percentages of oocytes with incomplete migration of cortical granules or incomplete mitochondrial congregation were significantly higher in oocytes matured at 40 °C than in oocytes matured at 37 °C (Table 3).

**Effects of heat stress on the intracellular glutathione levels of mouse oocytes**

Oocytes matured at 37 or 40 °C for 14 h were assayed for the total glutathione (GSH) and the GSSG levels, from which the level of GSH and the GSH/GSSG ratio were then calculated. Although, the total GSH did not differ between the two culture temperatures, the level of GSH was lower while that of GSSG was higher in oocytes matured at 40 °C than in oocytes cultured at 37 °C (Table 4). As a result, oocytes matured at 40 °C showed a significantly lower ratio of GSH/GSSG than oocytes matured at 37 °C. Together, the above results suggested that cytoplasmic maturation of mouse oocytes was affected when they were cultured at 40 °C.

**Effects of heat stress on spindle assembling and activation of spindle assembly checkpoint protein (MAD2) of mouse oocytes**

Spindle assembling and activation of spindle assembly checkpoint protein (MAD2) were examined in mouse oocytes matured under different temperatures for 14 h. Following α-tubulin staining, oocytes were classified into those with tine-pole spindles (Fig. 1E) and those with even-pole spindles (Fig. 1F) under a laser confocal microscope. After MAD2 labeling, some of the oocytes were MAD2 positive with labeling concentrated around the chromosomes (Fig. 1I) while others were MAD2 negative (Fig. 1J) when observed under a laser confocal microscope. Percentages of oocytes with tine-pole spindles did not differ between 37 and 40 °C, and no oocytes matured under these two temperatures were found MAD2-positive (Table 5). However, the percentage of oocytes with tine-pole spindles decreased significantly and over 20% of the oocytes became MAD2-positive after maturation at 40.7 °C. Furthermore, over 60% of the oocytes matured at 40.7 °C displayed scattered microtubule asters in the ooplasm (Fig. 1G). The results indicated that the damaging effect of heat stress on microtubule organization of mouse oocytes did not occur until the temperature increased to 40.7 °C.

**Discussion**

In this study, while rates of oocyte maturation did not differ from 37 to 40 °C, rates for blastocyst formation decreased significantly as maturation temperature increased from 38.5 to 39 °C. Likewise, while cytoplasmic maturation (migration of cortical granules and mitochondria and ratio of GSH/GSSG) was impaired at 40 °C, nuclear maturation (development to metaphase II) was not affected until 40.7 °C. Furthermore, chromosome spindle exchange showed that ooplasts matured at 40 °C failed to support blastocyst formation of reconstructed oocytes. The results strongly suggest that oocyte cytoplasmic maturation is less tolerant to heat stress than nuclear maturation. Fiorenza & Mangia (1992) reported that mild hyperthermic conditions (38.5–40 °C) during maturation disturbed the process of bivalent chromosome disjunction and blocked mouse oocytes at the metaphase I stage. This is in agreement with the present results that heat stress affected spindle assembling while activated the spindle assembly.
checkpoint protein MAD2. Roth & Hansen (2005) found that while bovine oocytes matured at 38.5 °C were mostly at metaphase II stage, the majority of heat-shocked oocytes were blocked at the first metaphase, first anaphase or first telophase stages. In addition, Roth & Hansen (2004) showed that exposure of bovine oocytes to thermal stress during the first 12 h of maturation reduced cleavage rate and the number of oocytes developed during the blastocyst stage. Tseng et al. (2006) found that rates for blastocyst formation of pig oocytes were reduced after post-maturation heat shock. However, thermotolerance was not compared between oocyte cytoplasmic and nuclear maturation. Edwards & Hansen (1996) found that exposure of bovine cumulus oocyte complexes to 41.8 °C did not alter the number of embryos that cleaved but reduced the number that developed to the blastocyst stage. Ju et al. (2005) reported that there were no significant differences in cleavage rates, but blastocyst formation and total cell number per blastocyst were lower in the post-maturation heat shock group compared with the control of bovine oocytes. The present results showed that blastocyst formation was normal when spindles matured at 40.7 °C were transplanted into in vivo matured ooplasts, although spindle assembling was obviously affected and MAD2 was activated in oocytes matured at 40.7 °C. However, when the in vivo matured spindles were transferred into ooplasts matured at 40 °C, no blastocyst formation was observed in reconstructed oocytes, apparently due to the impaired cytoplasmic maturation. This indicates that it is the cytoplasmic components but not the nuclear components that determine the pre-implantation developmental competence of an oocyte. Similarly, while transfer of the spindle apparatus from in vitro-grown and -matured mouse oocytes (that developed poorly when fertilized in vitro) to the in vivo matured ooplats resulted in a high rate of blastocyst development, transfer of the spindle apparatus from in vivo-matured oocytes into the in vitro-grown and -matured ooplasms produced poor quality embryos with a low rate of blastocyst formation (Liu et al. 2003a). Furthermore, while mouse oocytes reconstructed from fresh spindle apparatus and aged cytoplasm developed to blastocyst at

Table 3 Distribution of cortical granules and mitochondria in mouse oocytes matured under different temperatures.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Percentage of oocytes with incomplete migration of cortical granules</th>
<th>Percentage of oocytes with incomplete mitochondrial congregation</th>
</tr>
</thead>
<tbody>
<tr>
<td>37</td>
<td>17.3 ± 5.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>16.6 ± 0.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>40</td>
<td>50.4 ± 4.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>58.3 ± 0.9&lt;sup&gt;b&lt;/sup&gt;</td>
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</table>

<sup>a,b</sup>Values with a common letter in their superscripts did not differ (P>0.05). Each treatment was repeated three times and each replicate consisted of 20 oocytes.

Figure 1 Laser confocal micrographs (merged pictures) of mouse oocytes matured under different temperatures. While the fluorescence of FITC and Rhodamine 123 was pseudo-colored green, the fluorescence of Hoechst and PI was pseudo-colored blue and red respectively. Micrographs (A) and (B) are oocytes with complete and incomplete migration of cortical granules respectively. Micrographs (C) and (D) are oocytes with incomplete and complete congregation of mitochondria respectively. Micrographs (E), (F), and (G) are oocytes with tine-pole spindles, even-pole spindles, and scattered microtubule asters respectively. Micrograph H is an oocyte at the germinal vesicle-stage showing intensive MAD2 labeling around chromatin. Micrographs I and J are matured oocytes with MAD2 positive and negative respectively. First polar bodies are sometimes seen (arrows). The scale bar is 20 μm.
a low rate following fertilization or parthenogenetic activation, blastocyst formation rate of reconstructed oocytes from aged spindle apparatus and fresh cytoplasm was high (Bai et al. 2006). Taken together, the results substantiate the notion that while the nuclear (chromosomal or genetic) constitution determines the viability and morbidity of the late-stage fetus and offspring, the cytoplasmic constitution of the oocyte determines fertilization and the early development capacity of the embryo (Moor et al. 1998).

Our immunofluorescence microscopy revealed that hyperthermia during in vitro maturation impaired migration of both cortical granules and mitochondria of mouse oocytes. Previous studies have shown that migration of cortical granules to the egg cortex in the mouse was a continuous process regardless of germinal vesicle breakdown and it was normally completed before or soon after germinal vesicle breakdown (Ducibella & Buetow 1994, Liu et al. 2003b, 2005). Nishi et al. (2003) found that the rate of mitochondrial segregation was significantly higher in the in vivo than in vitro matured mouse oocytes. Miki et al. (2006) and Ge et al. (2008) reported that mouse oocytes matured under optimized conditions essentially resembled in vivo matured oocytes in mitochondrial distribution. According to Sun & Schatten (2006), while peripheral cortical granule migration in oocytes is controlled by microfilaments, mitochondria movement is mediated by microtubules. Therefore, it is suggested that the incomplete congregation of mitochondrial distribution observed in the heat-stressed mouse oocytes could have resulted from a poor mediation of mitochondrial translocation by the microtubules, because both the present study and others (Ju et al. 2005, Roth & Hansen 2005) have observed disturbed microtubule assembly following hyperthermia during in vitro maturation. The incomplete migration of cortical granules in heat stressed oocytes could have been due to a poor mediation by microfilaments, because alterations in microfilament structures were observed following heat stress of matured porcine oocytes (Ju & Tseng 2004). However, we were unable to find any difference in microfilament distribution between mouse oocytes matured at 37 and 40°C following staining with Phalloidin-TRITC (data not shown).

The present results indicated that heat shock during in vitro maturation decreased the GSH/GSSG ratio of mouse oocytes, although the total GSH level was similar between the heat stressed oocytes and unstressed controls. A positive effect of GSH concentration on oocyte developmental competence has been demonstrated, and therefore the intracellular GSH level is used as a marker for oocyte cytoplasmic maturation (de Matos et al. 1995, 2002, Abeydeera et al. 1998, Gasparini et al. 2003). Hyperthermia-induced oxidative stress has been suggested as one of the mechanisms by which heat stress disrupts reproductive performance. For example, exposing zygotes to heat shock has resulted in early embryonic loss, in association with increased hydrogen peroxide concentrations and reduced levels of GSH within the embryos (Ozawa et al. 2002). Hyperthermia enhances the production of ROS in the mouse liver (Ozawa et al. 2004) and oviduct (Matsuzuka et al. 2005), shifting the redox status toward oxidative stress. In addition, in vitro supplementation of GSH or GSH ester reduced the effect of heat shock on viability of mouse embryos (Aréchiga et al. 1995), while inhibition of GSH synthesis aggravated the deleterious effect of heat stress on the oocyte developmental capacity (Edwards & Hansen 1997). Furthermore, treatment of buffalo–cows or supplementation with antioxidants before the beginning of months of heat-stress and during the stress period corrected the infertility due to heat-stress through the decrease in cortisol secretion and a decrease in the oxidative stress (Megahed et al. 2008). Similarly, pretreatment of female mice with antioxidants alleviated the negative effect of hyperthermia on developmental competence of oocytes and improve embryonic development (Roth et al. 2008).

**Table 4** GSH levels (pmol/oocyte) in mouse oocytes matured under different temperatures.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Total GSH</th>
<th>GSH</th>
<th>GSSG</th>
<th>GSH/GSSG</th>
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</thead>
<tbody>
<tr>
<td>37</td>
<td>5.75±4.35&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.02±0.67&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.33±2.63&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.24±2.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>40</td>
<td>5.73±2.67&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.69±2.15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.39±2.72&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.13±4.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a,b</sup>Values with a common letter in their superscripts did not differ (P>0.05).

**Table 5** Spindle assembling and activation of spindle assembly checkpoint protein (MAD2) in mouse oocytes matured under different temperatures.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Percentage of oocytes with tine pole spindles</th>
<th>Percentage of oocytes with microtubule asters</th>
<th>Percentage of MAD2 positive oocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>37</td>
<td>73.1±4.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.0±0.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.0±0.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>40</td>
<td>77.0±3.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.0±0.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.0±0.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>40.7</td>
<td>33.7±8.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>61.7±8.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>22.6±7.4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a,b</sup>Values with a common letter in their superscripts did not differ (P>0.05). Each treatment was repeated three times and each replicate consisted of 20 oocytes.
In this study, while the percentage of oocytes with tine-pole spindles did not differ from 37 to 40 °C, it decreased significantly in oocytes matured at 40.7 °C. The barrel-shaped spindle has been considered less normal than the tine-pole spindle and it occurred more frequently in the *in vitro* matured than *in vivo* matured oocytes (Sanfins *et al.* 2003). Ge *et al.* (2008) found that while the percentage of oocytes with tine-pole spindles was lower, that of oocytes with even-pole spindles was higher significantly following maturation with cumulus denuded than with cumulus intact. Roth & Hansen (2005) noticed that a subset of bovine oocytes possessed misshapen metaphase-I spindles with disorganized microtubules and unaligned chromosomes after heat-shock during *in vitro* maturation. Ju *et al.* (2005) found that the metaphase spindle became elongated or aberrant and smaller following post-maturation heat shock of bovine oocytes. In addition, we observed microtubule organizing center-like structures in some of the oocytes matured at 40.7 °C, but not in oocytes matured at 37 or 40 °C. Ju & Tseng (2004) also showed that the spindle microtubules of porcine oocytes were completely depolymerized or formed as microtubule arrays following post-maturation heat stress. Furthermore, our recent observations indicated that while freshly ovulated oocytes did not, 80% of the aged mouse oocytes formed cytoplasmic asters of microtubules (data to be published). The results that heat stress during *in vitro* maturation impaired microtubule organization were further substantiated by our observation that spindle assembly checkpoint protein (MAD2) was activated in some of the mouse oocytes matured at 40.7 °C. Zhang *et al.* (2004) found that MAD2 was at the kinetochores in rat oocytes at germinal vesicle and prometaphase I stages, but disappeared once the oocytes reached the metaphase I or metaphase II stage. Similarly, while no MAD2 was observed in freshly ovulated mouse oocytes in this study, a MAD2 concentration around chromosomes was obvious in heat-stressed mouse oocytes. However, when rat oocytes at metaphase II stage were treated by nocodazole, spindles were destroyed and MAD2 reappeared (Zhang *et al.* 2004). This suggests that heat stress destroyed the spindles and thereby activated MAD2 of mouse oocytes.

**Materials and Methods**

Chemicals were purchased from Sigma Chemical Co. unless and otherwise specified.

**Animals and oocyte recovery**

**Animals**

Mice of the Kunming breed were kept in a room with 14h light:10h darkness cycles, the darkness starting from 2000 h.

The animals were handled by the rules stipulated by the Animal Care and Use Committee of Shandong Agricultural University.

**In vivo matured oocytes**

Female mice, 6–8 weeks after birth (weighing around 30 g), were induced to superovulate with eCG (10 IU, i.p.) followed 48 h later by hCG (10 IU, i.p.). Both eCG and hCG used in this study were from Ningbo Hormone Product Co., Ltd, Hangzhou city, P.R. China. The superovulated mice were killed at 15 h after hCG injection (Fig. 2) and the oviductal ampullae were broken in M2 medium to release ovulated oocytes.

**Germinal vesicle stage oocytes**

Female mice, 3–4 weeks after birth (weighing about 20 g), were killed at 48 h after eCG administration, and the large follicles on the ovary were ruptured in M2 medium to release oocytes at the germinal vesicle stage. Only the oocytes that were surrounded with more than three layers of unexpanded cumulus cells and had a diameter of >70 μm and a homogenous cytoplasm were selected for *in vitro* maturation culture.

**In vitro maturation of oocytes**

Oocytes at the germinal vesicle stage were washed three times in M2 medium and once in maturation medium. After washing, the oocytes were placed in 100 μl drops maturation medium (around 30 per drop) and cultured for 14 h at different temperatures in a humidified atmosphere of 5% CO2 in air (Fig. 2). The maturation medium was TCM-199 (Gibco) supplemented with 10% (v/v) FCS (Gibco), 1 μg/ml 17β-estradiol, 24.2 mg/l sodium pyruvate, 0.05 IU/ml FSH, 0.05 IU/ml LH and 10 ng/ml EGF. To reduce variation of temperature during oocyte culture, two CO2 incubators of the same type (CO2 Water Jacketed Incubator Series II, Model: 3110 Series; Forma Scientific Inc., Marietta, OH, USA) were used; while one incubator was always set at 37 °C, the other was used for different temperatures. The inside temperature of the two incubators was used for different temperatures. The inside temperature of the two incubators was used for different temperatures.

**Figure 2** A time-line figure illustrating the experimental setup. Oocytes at the germinal vesicle-stage were cultured for 14 h at different temperatures to obtain *in vitro* matured (IVM) oocytes. In *in vivo* matured (IVO) oocytes were collected from superovulated female mice at 15 h post-hCG injection. Spindle transfer (ST), which took about 2 h, was performed immediately after collection of mature oocytes. After maturation culture or ST, the IVM oocytes and oocytes reconstructed with IVM ooplasm were aged for 8–10 h at 37 °C in the maturation medium, before parthenogenetic activation (PA) treatment, while the oocytes reconstructed with IVO ooplasm were treated for PA immediately after ST.
an incubator was monitored by simultaneously examining three clinical thermometers placed inside the incubator. Every time to change the temperature, the temperature inside the incubator was checked for constancy every 6 h for at least 1 day before oocyte culture. The door of incubator was kept closed during the whole maturation period (14 h), and the temperature inside was examined again at the end of culture.

**Chromosome spindle transfer**

Oocytes at the metaphase II stage of either *in vitro* or *in vivo* origin were stripped of their cumulus cells by pipetting in M2 medium containing 0.1% hyaluronidase. Denuded oocytes were treated for 15 min in the micromanipulating medium (M2 containing 5 mg/ml cytochalasin B) before micromanipulation. The micromanipulation procedures were carried out using a Leica differential interference contrast microscope, under which the mouse spindle was identifiable as a translucent region. An injection pipette with a flattened tip and an inner diameter of 15–18 μm, driven by a piezo system, was used for both removal and injection of the metaphase II chromosome spindles. The metaphase II spindle with a small amount of ooplasm was pinched off from each oocyte. Isolated karyoplasts from heat stressed oocytes were inserted into the perivitelline space of enucleated non-stressed oocytes while those from the non-stressed oocytes were inserted into the enucleated stressed oocytes.

After rinsing in M2, the karyoplast–cytoplast pairs were equilibrated in fusion medium (0.3 M mannitol, 0.1 mM MgSO4, 0.1 mg/ml polyvinyl alcohol and 3 mg/ml BSA) for 3 min. Fusion between karyoplasts and cytoplasts was then induced with a single DC pulse of 1.0 kV/cm for 30 μs, using a BEX LF201 (Nepa Gene Co., Ltd, Shioyaki Ichikawa Chiba, Japan) with two microelectrodes. Thirty minutes later, fused karyoplast–cytoplast pairs were selected for activation treatment.

**Oocyte activation and embryo culture**

Procedures used for oocyte activation were those reported by Ma et al. (2005) with a few modifications. The activating medium used was Ca2+-free Chatot–Ziomek–Bavister (CZB) medium supplemented with 10 mM SrCl2. After maturation culture or spindle transfer, the *in vitro* matured oocytes and oocytes reconstructed with *in vitro* matured ooplasms were aged for 8–10 h at 37 °C in the maturation medium, before activation treatment, while the oocytes reconstructed with *in vivo* matured ooplasms were treated for activation immediately after spindle transfer (Fig. 2). Oocytes were stripped of their cumulus cells, if any, by pipetting in M2 containing 0.1% hyaluronidase. After being washed twice in M2 and once in activating medium, the oocytes were incubated first in activating medium for 2.5 h and then in regular CZB without SrCl2 for 3.5 h at 37 °C in a humidified atmosphere with 5% CO2 in air. Both the activating medium and CZB for subsequent short culture of oocytes were supplemented with 5 μg/ml cytochalasin (CB) to diploidize the parthenotes. Six hours after the onset of activation treatment, the oocytes were examined with a microscope for the evidence of activation. Oocytes were considered activated when each contained one (1PN) or two well developed pronuclei (2PN).

The Sr2+-activated oocytes were cultured for 4 d in the regular CZB without CB (20 embryos per 60 μl drop) at 37.5 °C under humidified atmosphere with 5% CO2 in air. Glucose (5.5 mM) was added to CZB when 2/3 of the embryos cultured and developed to the 4-cell stage. Assessment of embryo development was performed at 24 h (2-cell stage), 48 h (4-cell stage), and 96 h (blastocyst) after the onset of activation. Some of the blastocysts were mounted on a slide, stained with Hoechst 33342 and observed for cell counts under a fluorescence microscope.

**Assay for intracellular GSH**

Intracellular content of GSH was measured as described by Funahashi et al. (1994). Cumulus-free oocytes were washed three times in M2. Five micro-liters of distilled water containing 35–40 oocytes was transferred to a 1.5 ml microfuge tube, and then 5 μl of 1.25 M phosphoric acid were added to the tube. Samples were frozen at −80 °C and thawed at room temperature. This procedure was repeated three times. Then, the samples were stored at −20 °C until analyzed. Concentrations of total GSH in the oocyte were determined by the 5, 5′-dithio-bis-(2-nitrobenzoic acid) (DTNB)-oxidized glutathione (GSSG) reductase-recycling assay. Briefly, 700 μl of 0.33 mg/ml NADPH in 0.2 M sodium phosphate buffer containing 10 mM EDTA (stock buffer, pH 7.2), 100 μl of 6 mM DTNB in the stock buffer, and 190 μl distilled water were added and mixed in a microfuge tube. Ten micro-liters of 250 IU/ml GSH reductase (G-3664) were added with mixing to initiate the reaction. The absorbance was monitored continuously at 412 nm with a spectrophotometer for 3 min, with reading recorded every 0.5 min. To measure the concentrations of GSSG, the samples (10 μl) were vigorously mixed with 0.2 μl 2-vinylpyridine and 0.6 μl triethanolamine. After 60 min, the sample was assayed as described above in the DTNB-GSSG reductase-recycling assay. Standards (0.01, 0.02, 0.1, 0.2, and 1.0 mM) of GSH and a sample blank lacking GSH were also assayed. The amount of GSH in each sample was divided by the number of oocytes to get the intracellular GSH concentration per oocyte. The GSH values were calculated from the difference between total (GSSG + GSH) and GSSG for each oocyte and expressed as pmol/oocyte.

**Immunofluorescence microscopy**

**Cortical granule staining**

Zona pellucida was removed by treating oocytes with 0.5% pronase (Roche) in M2. After being washed twice in a washing solution (M2 supplemented with 0.3% BSA and 0.01% Triton X-100), oocytes were fixed with 3.7% paraformaldehyde in M2 for 30 min at room temperature. The oocytes were then blocked three times for 5 min each in a blocking solution (M2 containing 0.3% BSA and 100 mM glycine). After
permeabilization for 5 min in M2 containing 0.1% Triton X-100, oocytes were washed twice again for 5 min each in blocking solution. They were then cultured in 100 μg/ml FITC (FITC)-labeled lens culinaris agglutinin in M2 for 30 min in the darkness. Finally, the oocytes were washed three times in the washing solution, and stained with 10 μg/ml PI for chromatin examination.

Mitochondria staining

Denuded oocytes were washed in M2 and incubated in 10 μg/ml Rhodamine 123 in M2 at 37 °C for 15 min. Then, the oocytes were washed three times in M2 and stained for 5 min at room temperature with 10 μg/ml Hoechst 33342 in M2.

Spindle (α-tubulin) staining

Cumulus-free oocytes were fixed with 4% formaldehyde in PHEM buffer (60 mM, 25 mM Hapes, 10 mM EGTA, 4 mM MgSO4, PH 7.0) for 20 min at room temperature. The fixed oocytes were rinsed three times for a total of 15 min in PBS and then treated for 10 min in 1% Triton-X100 in PHEM. After they were briefly washed in PBS, oocytes were blocked in 20% goat serum in PHEM at 4 °C overnight. Fixed oocytes were incubated for 1 h at room temperature in PHEM containing FITC-conjugated monoclonal anti-α-tubulin (1:50) and 5% goat serum. The oocytes were then washed and stained with 10 μg/ml Hoechst 33342 for chromosome examination.

MAD2 staining

Cumulus-free oocytes were fixed in 4% paraformaldehyde/PHEM (60 Mm Pipes, 25 mM Hapes at pH 6.9, 10 mM EGTA, 8 mM MgSO4, PH 7.0) for 20 min and washed three times in PBS containing 0.05% PVP. The oocytes were then treated in 0.5% Triton X-100/PHEM for 5 min and washed rapidly three times in PBS with 0.05% PVP. After being blocked in 1% BSA/PHEM with 100 mM glycine at room temperature for 1 h, the oocytes were incubated in anti-MAD2 antibody (1:100 in 1% BSA/PHEM with 100 mM glycine) at 4 °C overnight. After four washes in PBS with 0.05% Tween 20, the oocytes were incubated with FITC-conjugated goat-anti-rabbit IgG (1:200 in 1% BSA/PHEM with 100 mM glycine) for 45 min. After three washes in PBS with 0.05% Tween 20, the oocytes were stained with PI (10 μg/ml) for examination of chromosomes.

Laser confocal microscopy

After washing, the stained oocytes were mounted on glass slides and observed with a Leica laser scanning confocal microscope. Hoechst 33342 labeled nuclear chromatin was excited with the 405 nm line of a diode laser. The FITC, PI, and Rhodamine 123 fluorescence was obtained by excitation with 488 nm line of an Ar/ArHr laser and the emitted light was passed through a 488 nm filter. The individual optical sections were pseudo-colored and digitally recombined into a single composite image using the Leica Confocal Software.

Data analysis

We conducted at least three replicate trials for each treatment. The percentage data were arc sine transformed and the transformed data were tested to verify ANOVA assumptions (normally distributed and homogenous variance) before being analyzed with ANOVA. A Duncan multiple comparison test was used to locate differences. The software used was SPSS (Statistical Package for Social Sciences, version 11.5, SPSS Inc., Chicago, IL, USA). Data were expressed as means ± S.E.M. and P<0.05 was considered significant.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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