Pyruvate prevents aging of mouse oocytes

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Abstract

Inhibiting oocyte aging is important not only for healthy reproduction but also for the success of assisted reproduction techniques. Although our previous studies showed that cumulus cells accelerated aging of mouse oocytes, the underlying mechanism is unknown. The objective of this paper was to study the effects of pyruvate and cumulus cells on mouse oocyte aging. Freshly ovulated mouse cumulus–oocyte complexes (COCs) or cumulus-denuded oocytes (DOs) were cultured in Chatot-Ziomek-Bavister (CZB) medium or COC-conditioned CZB medium supplemented with different concentrations of pyruvate before being examined for aging signs and developmental potential. Pyruvate supplementation to CZB medium decreased rates of ethanol-induced activation in both COCs and DOs by maintaining their maturation-promoting factor activities, but more pyruvate was needed for COCs than for DOs. Addition of pyruvate to the COC-conditioned CZB also alleviated aging of DOs. Observations on cortical granules, level of BCL2 proteins, histone acetylation, intracellular concentration of glutathione, and embryo development all confirmed that pyruvate supplementation inhibited aging of mouse oocytes. It is concluded that the aging of mouse oocytes, facilitated by culture in COCs, can be partially prevented by the addition of pyruvate to the culture medium.


Introduction

Mammalian oocytes are arrested at meiotic metaphase II (M II) following ovulation or in vitro maturation until they are activated by penetrating spermatozoa or artificial stimuli. Persistently high levels of the maturation-promoting factor (MPF) activities were found to be necessary for the maintenance of M II arrest (Smith & Ecker 1970, Masui & Markert 1971, Wu et al. 1997). If not fertilized or artificially activated in time, the mature oocytes undergo a time-dependent process of aging (Yanagimachi & Chang 1961, Whittingham & Siracusa 1978). This process of oocyte aging has been found to be always associated with a decrease in the MPF activity (Xu et al. 1997, Abbott et al. 1998, Miao et al. 2005). Furthermore, experimental regulation of the MPF activity showed that the deteriorating changes in aged oocytes such as enhanced activation and higher fragmentation rates following parthenogenetic activation could be attributed to the gradual decrease in MPF activity during oocyte aging (Kikuchi et al. 2000).

Fertilization of postovulatory aged oocytes gives rise to mice suffering from nervous and emotional abnormalities (Tarin et al. 1999) and decreased reproductive fitness and longevity (Tarin et al. 2002). Humans and some animals potentially undertake sexual activity on any day of the estrous cycle, which may cause fertilization of aged ovulated oocytes. Like detention in the oviduct, in vitro culture of matured oocytes also led to oocyte aging (Longo 1980, Webb et al. 1986, Tan 1988). Many experimental designs in both research and clinical applications involve culture of matured oocytes prior to micromanipulation or insemination. For example, the cell cycle stage of the recipient cytoplasts must be finely controlled for the success of nuclear transfer for cloning (Zhou et al. 2003, Wu et al. 2007), and rates of cell fusion and embryonic development of nuclear transfer embryos decreased significantly when aged oocytes were used for recipient cytoplasts (Cervera & García-Ximénez 2003, Iwamoto et al. 2005). Therefore, studies on the mechanisms and control of oocyte aging are important for the healthy reproduction of both humans and nonhuman mammals.
Knowledge of the interactions between the germ and somatic cells is an essential part of the cell-cell interactions in cell biology. The role of the surrounding cumulus cells in maturation, ovulation, and fertilization of oocytes has been extensively studied (Eppig 1982, 1991, Buccione et al. 1990, Tanghe et al. 2002); yet little is known about their role in oocyte aging. Moreover, although the cumulus cells have been found to play an important role in maturation, ovulation, and fertilization of oocytes, their mechanisms of action are poorly understood (Tanghe et al. 2002, Ge et al. 2007). In recent years, two studies were conducted in this laboratory to investigate the role and mechanisms of action of cumulus cells in mouse oocyte aging. Our first study demonstrated that during in vitro aging of mouse cumulus-oocyte complexes (COCs), activation rates increased while the MPF activity decreased significantly as during in vivo aging of the ovulated oocytes (Miao et al. 2005). During aging of cumulus-denuded oocytes (DOs), however, activation rates remained low and the MPF activity decreased much more slowly compared with that of oocytes aged with cumulus cells. Our second study showed that both co-culture with COCs or monolayer of cumulus cells and culture in medium conditioned with these cells promoted activation of DOs (Qiao et al. 2007). The results strongly suggest that cumulus cells accelerate aging of mouse oocytes, most probably by secreting a soluble factor(s). However, the aging-promoting factor remains to be characterized to understand interactions between the aging cells and thereby the mechanisms for oocyte and somatic cell aging.

According to Leese & Barton (1984), in the absence of cumulus cells, pyruvate uptake exceeded that of glucose in unfertilized and fertilized ova and in the developmental stages up to the blastocyst, when glucose became the predominant substrate. Sutton et al. (2003) showed that when metabolic activity for the bovine COCs was analyzed relative to time in culture, there was an approximate twofold increase in the consumption of oxygen, glucose, and pyruvate over the 24 h period, while the metabolic activity of DOs was undetectable. Furthermore, Downs et al. (2002) demonstrated that 5–6 times more pyruvate was consumed by COCs than by DOs during maturation in the absence of glucose. It can be seen from these results that the matured oocytes are capable of metabolizing pyruvate and that COCs consume much more pyruvate than DOs do in the absence of glucose. We therefore hypothesize that oocytes would age quickly in the absence of pyruvate, and cumulus cells would accelerate oocyte aging by depleting pyruvate in the Chatot-Ziomek-Bavister (CZB) medium that does not contain glucose. The objective of the present study was to test this hypothesis and find out whether supplementation of pyruvate in culture medium would prevent oocyte aging.

**Results**

**Pyruvate supplementation decreased activation while increased MPF activity of aging mouse oocytes**

Freshly ovulated mouse COCs or DOs were cultured for 6 h in CZB medium supplemented with pyruvate at different concentrations. At the end of the culture, oocytes were either treated with ethanol for activation or assayed for MPF activity. When COCs were cultured in CZB containing low levels (0.27 or 2.5 mM) of pyruvate, activation rates increased while MPF activity decreased significantly compared with those of freshly ovulated oocytes in the control group (Table 1). When COCs were cultured in CZB supplemented with higher concentrations (5, 10, or 20 mM) of pyruvate, however, activation rates and MPF activity did not change as much as those in control oocytes. The activation rate and MPF activity of DOs cultured without extra pyruvate was close to those of control oocytes. The results indicated that pyruvate inhibited aging of mouse oocytes by maintaining their MPF activity and that more pyruvate was required to inhibit aging of COCs than DOs.

**Pyruvate supplementation improved fertilizability and developmental capacity of aged oocytes**

To study the effect of pyruvate supplementation on fertilizability and zona hardening of aging oocytes, freshly ovulated mouse COCs were cultured for 6 or 12 h in CZB medium supplemented with or without 10 mM pyruvate. At the end of culture, oocytes were either in vitro inseminated or subjected to chymotrypsin digestion assay of zona pellucida (ZP). While percent-ages of fertilized oocytes decreased, the chymotrypsin digestion time (t50, min) of ZP increased significantly with time of oocyte aging (Table 2). Supplementation of pyruvate significantly increased fertilizability and zona hardening of aged COCs.

<table>
<thead>
<tr>
<th>CZB supplemented with pyruvate (mM)</th>
<th>COC/DO</th>
<th>Oocytes observed</th>
<th>Percentage of oocytes fertilized</th>
<th>MPF activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>89</td>
<td>13.3 ± 2.8a</td>
<td>100.0 ± 0.0a</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>COC</td>
<td>133</td>
<td>71.2 ± 1.9b</td>
<td>54.7 ± 3.6b</td>
</tr>
<tr>
<td>0</td>
<td>DO</td>
<td>100</td>
<td>12.0 ± 1.7a</td>
<td>92.2 ± 3.2a</td>
</tr>
<tr>
<td>2.5</td>
<td>COC</td>
<td>97</td>
<td>50.4 ± 1.0a</td>
<td>61.3 ± 3.4b</td>
</tr>
<tr>
<td>5</td>
<td>COC</td>
<td>133</td>
<td>16.6 ± 2.9a</td>
<td>98.5 ± 2.9a</td>
</tr>
<tr>
<td>10</td>
<td>COC</td>
<td>138</td>
<td>5.0 ± 2.4d</td>
<td>115.5 ± 3.9c</td>
</tr>
<tr>
<td>20</td>
<td>COC</td>
<td>106</td>
<td>2.0 ± 1.3d</td>
<td>120.1 ± 4.2c</td>
</tr>
</tbody>
</table>

*a,b,c*Values with a common letter in their superscripts did not differ (P > 0.05) in the same column.

In each treatment, three samples collected on different experimental days were assayed for the relative activity of H1 kinase and each sample contained 40 oocytes.

**Table 1** Ethanol-induced activation and maturation-promoting factor (MPF) activity after freshly ovulated cumulus-oocyte complexes (COCs) or cumulus-denuded oocytes (DOs) were cultured in vitro for 6 h in CZB medium supplemented with different concentrations of pyruvate.

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Freshly ovulated control

Control 0

In vivo aging

6

12

In vitro aging

6

12

Treatment supplementation were inseminated following zona drilling. Fertilization and embryo development after mouse cumulus–oocyte complexes that had been aged in CZB with or without pyruvate supplementation were classed according to the gray level into heavily stained (HS), with a gray level value of 100–170 (Fig. 1D); moderately stained (MS), with a gray level value of 50–100 (Fig. 1E); and not stained (NS), with a gray level value of 0–25 (Fig. 1F). While all of the freshly ovulated control oocytes underwent cortical granule exocytosis, all the oocytes aging in vivo or in vitro without pyruvate supplementation showed either moderate or heavy cortical granule exocytosis (Table 4). When cultured in the presence of extra pyruvate, however, only a few oocytes underwent moderate and none underwent HE of cortical granules.

Pyruvate supplementation increased the level of anti-apoptotic protein BCL2 while decreased the level of histone acetylation in aged oocytes

Freshly ovulated COCs or DOs were cultured for 12 h in CZB medium supplemented with or without 10 mM pyruvate before being labeled with antibodies against BCL2 proteins or acetylated lysine 12 of histone H4 (H4K12) and lysine14 of histone H3 (H3K14). Following BCL2 staining, oocyte images, obtained using a confocal microscope under the same exposure conditions, were analyzed for gray level using Image-Pro Plus software (Media Cybernetics Inc., Bethesda, MA, USA). Oocytes were classified according to the gray level into heavily stained (HS), with a gray level value of 100–170 (Fig. 1D); moderately stained (MS), with a gray level value of 50–100 (Fig. 1E); and not stained (NS), with a gray level value of 16–50 (Fig. 1F). While all of the freshly ovulated control oocytes were HS with BCL2 antibodies, none of the oocytes aging in vivo or in vitro without pyruvate supplementation was HS (Table 5). When cultured in the

Table 3 Fertilization and embryo development after mouse cumulus–oocyte complexes that had been aged in CZB with or without pyruvate supplementation were inseminated following zona drilling.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Aging time (h)</th>
<th>Pyruvate (10 mM)</th>
<th>Oocytes inseminated</th>
<th>Percentage of oocytes fertilized</th>
<th>Percentage of blastocysts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>—</td>
<td>109</td>
<td>93.1 ± 1.7a</td>
<td>79.0 ± 1.8a</td>
</tr>
<tr>
<td>In vivo</td>
<td>6</td>
<td>—</td>
<td>108</td>
<td>82.1 ± 3.1b</td>
<td>69.1 ± 1.2b</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>—</td>
<td>133</td>
<td>55.6 ± 5.5c</td>
<td>39.9 ± 1.9c</td>
</tr>
<tr>
<td>In vitro</td>
<td>6</td>
<td>—</td>
<td>109</td>
<td>78.4 ± 3.2d</td>
<td>67.3 ± 2.0d</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>+</td>
<td>110</td>
<td>84.6 ± 1.3e</td>
<td>76.3 ± 3.1e</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>—</td>
<td>130</td>
<td>52.2 ± 5.5f</td>
<td>32.7 ± 1.1f</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>+</td>
<td>129</td>
<td>81.6 ± 2.1b</td>
<td>59.8 ± 2.8d</td>
</tr>
</tbody>
</table>

a–bValues with a common letter in their superscripts did not differ (P > 0.05) in the same column.
presence of extra 10 mM pyruvate, however, proportions of labeled oocytes increased while those of not labeled oocytes decreased significantly.

Following labeling with antibodies against acetylated histone, oocytes were classified as those with chromatin stained (Fig. 1G and H) and those with chromatin unstained (Fig. 1I). Acetylation of either H4K12 or H3K14 was detected in none of the freshly ovulated oocytes (Table 5). While 100% of the oocytes showed acetylated H4K12 and 75% showed acetylated H3K14 when aging without extra pyruvate, few or no oocytes showed acetylated H4K12 and H3K14 respectively, when aging in the presence of extra 10 mM pyruvate.

Effects of pyruvate supplementation on the intracellular GSH levels of aged oocytes

Oocytes aging for 12 h in CZB supplemented with or without 10 mM pyruvate were assayed for the total GSH and the oxidized glutathione (GSSG) levels, from which the level of GSH and the GSH/GSSG ratio were then calculated. While both the total GSH level and the GSH level decreased, the GSSG level increased in oocytes aged in vivo or in vitro without pyruvate supplementation compared with freshly ovulated controls (Table 6). As a result, oocytes aged without extra pyruvate showed a significantly lower ratio of GSH/GSSG than control oocytes.
Pyruvate supplementation increased the GSH/GSSG ratio in aged oocytes mainly by increasing the level of GSH while having a mild effect on the GSSG level.

**Pyruvate supplementation counteracted the oocyte aging-accelerating effect of medium conditioned with cumulus cells**

Two experiments were conducted to test the hypothesis that cumulus cells accelerate oocyte aging by depleting pyruvate from the culture medium. In the first experiment, freshly ovulated DOs were cultured for 6 h in COC- or DO-conditioned CZB medium supplemented with or without pyruvate before being treated for activation or assayed for MPF activity. When cultured in COC-conditioned CZB without pyruvate supplementation, activation rates of DOs increased while their MPF activity decreased significantly compared with control oocytes cultured in unconditioned CZB (Table 7). Results in DO-conditioned CZB medium were similar to controls. When cultured in COC-conditioned CZB supplemented with 10 mM pyruvate, both activation rates and MPF activity remained close to those of control oocytes cultured in unconditioned or DO-conditioned CZB medium.

In the second experiment, pyruvate and lactate concentrations in freshly prepared CZB and CZB that had been conditioned with COCs or DOs for 6 or 12 h were analyzed by capillary electrophoresis. To our surprise, instead of decreasing, the pyruvate concentration measured in the COC-conditioned CZB increased significantly with time compared with that in fresh CZB (Table 8). The concentration of lactate in the COC-conditioned CZB decreased with time. The CZB conditioned with DOs showed pyruvate and lactate concentrations similar to those in fresh CZB.

**Discussion**

The most prominent manifestations of aged oocytes include an increased susceptibility to activating stimuli (Kubiak 1989, Lan et al. 2004), a decrease in MPF activity (Wu et al. 1997, Xu et al. 1997), a partial exocytosis of cortical granules (Szollosi 1971, Ducibella et al. 1990), signs of apoptotic cell death such as increased cytoplasmic and DNA fragmentation (Gordo et al. 2000) and decreased levels of BCL2 proteins (Gordo et al. 2002), the onset of anaphase II (Xu et al. 1997, Abbott et al. 1998), and impaired embryo development following fertilization (Juetten & Bavister 1983, Tesarik 1993, Winston et al. 1993, Tarin et al. 1998). In addition, an increased acetylation on some lysines of histones has also been observed in aged mouse oocytes (Huang et al. 2007). The present results showed that pyruvate supplementation to the culture medium decreased the susceptibility to activation stimulus while increased the MPF activity of aged oocytes; it increased the level of the anti-apoptotic BCL2 and decreased levels of BCL2 proteins while increasing premature exocytosis of the cortical granules of the aged mouse oocytes.

Pyruvate supplementation also slowed down the acetylation of lysine 12 of histone H4 (H4K12) and lysine 14 of histone H3 (H3K14) and increased the rates of normal fertilization and blastocyst formation of aged oocytes.

**Table 4** Patterns of cortical granule distribution after freshly ovulated cumulus–oocyte complexes were aged for 12 h in CZB with or without pyruvate supplementation.

<table>
<thead>
<tr>
<th>Type of oocytes</th>
<th>Pyruvate (10 mM)</th>
<th>NE</th>
<th>ME</th>
<th>HE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freshly ovulated</td>
<td>−</td>
<td>100±0.0 b</td>
<td>0.0±0.0 a</td>
<td>0.0±0.0 a</td>
</tr>
<tr>
<td>In vivo aging</td>
<td>−</td>
<td>6±0.5</td>
<td>76.4±1.9 a</td>
<td>23.6±2.6 b</td>
</tr>
<tr>
<td>In vitro aging</td>
<td>−</td>
<td>0±0.5</td>
<td>71.2±2.3 b</td>
<td>28.8±3.4 b</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>91.5±3.7 a</td>
<td>8.5±4.2 c</td>
<td>0.0±0.0 a</td>
</tr>
</tbody>
</table>

a–cValues with a common letter in their superscripts did not differ significantly (P>0.05) in the same column. Each treatment was repeated thrice and each replicate contained 20 oocytes. NE, ME, and HE stand for no, moderate, and heavy exocytosis of cortical granules respectively.

**Table 5** Levels of BCL2 and histone acetylation after freshly ovulated cumulus–oocyte complexes were aged for 12 h in CZB with or without pyruvate supplementation.

<table>
<thead>
<tr>
<th>Type of oocytes</th>
<th>Pyruvate (10 mM)</th>
<th>Percentage of oocytes with different levels of BCL2</th>
<th>Percentage of oocytes with acetylated histone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>−</td>
<td>HS: 100±0.0 a</td>
<td>H4K12: 0.0±0.0 a</td>
</tr>
<tr>
<td>Freshly ovulated</td>
<td>−</td>
<td>MS: 0.0±0.0 a</td>
<td>H3K14: 0.0±0.0 a</td>
</tr>
<tr>
<td>In vivo aging</td>
<td>−</td>
<td>NS: 0.0±0.0 a</td>
<td></td>
</tr>
<tr>
<td>In vitro aging</td>
<td>−</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a–cValues with a common letter in their superscripts did not differ significantly (P>0.05) in the same column. Each treatment was repeated thrice and each replicate contained 20 oocytes. HS, MS, and NS stand for heavily, moderately, and not stained by anti-BCL2 antibodies respectively.

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Pyruvate and oocyte aging

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In addition, pyruvate supplementation increased the GSH/GSSG ratio of aged mouse oocytes. Kim & Schuetz (1991) observed spontaneous pronuclear formation following in vitro culture of mouse oocytes in pyruvate-deficient medium. Together, the results suggest that pyruvate inhibits aging of mouse oocytes.

The present results demonstrated that the MPF activity decreased quickly in mouse COCs aging in vitro in the absence of pyruvate supplementation. The MPF is a heterodimer formed of p34\(^\text{cd}\text{c}2\) protein kinase and cyclin B1/B2, and without cyclin B, the CDC2 kinase will not function (Gautier et al. 1990, Nurse 1990). It has been shown that the high activity of MPF in oocytes arrested at the M II stage is maintained through a continuous equilibrium between synthesis and degradation of cyclin B (Kubiak et al. 1993). Inhibition of protein synthesis through treatment with cycloheximide resulted in activation of mouse M II oocytes, as assessed by emission of the second polar body and formation of a pronucleus (Siracusa et al. 1978, Clarke & Masui 1983, Fulka et al. 1994). It was subsequently confirmed that a concomitant decrease in CDC2/cyclin B kinase activity occurred with the cycloheximide induced oocyte activation (Moos et al. 1996). Kim & Schuetz (1991) reported that protein synthesis was markedly inhibited when mouse oocytes were cultured in the pyruvate deficient medium. It is therefore postulated that the pyruvate insufficiency-induced oocyte activation occurs by affecting the metabolism of oocytes and hence inhibiting the synthesis of proteins including cyclin B that results in a decrease in the MPF activity.

This study showed that while the digestion time of ZP increased, percentages of fertilized oocytes decreased significantly in aged oocytes with either intact or drilled zona. Pyruvate supplementation blocked premature exocytosis of cortical granules, reduced the digestion time of ZP and increased fertilizability of aged oocytes. It is known that in all mammals, the ZP and egg plasma membrane work synergistically to prevent sperm entry, and the cortical granule contents released upon fertilization are involved in both the zona and plasma membrane block to polyspermy (Yanagimachi 1994). Premature spontaneous release of cortical granules in aged oocytes has long been documented (Szollosi 1971, Ducibella et al. 1990). The role of Ca\(^{2+}\) rise in egg activation and cortical granule release is well established in mouse (Tombes et al. 1992). A decreased intracytoplasmic level of ATP (Chi et al. 1988) and increased intracellular Ca\(^{2+}\) and reduction of the endoplasmic reticulum Ca\(^{2+}\) have been observed in aged oocytes, and the increase in intracellular Ca\(^{2+}\) has been attributed to dysfunction of the endoplasmic reticulum Ca\(^{2+}\)-ATPase in these oocytes (Vincent et al. 1992, Igarashi et al. 1997, Takahashi et al. 2000). Moreover, our recent experiments showed that rotenone, a mitochondrial inhibitor, accelerated mouse oocyte aging in the presence of pyruvate (data to be published). Therefore, the mechanism by which pyruvate blocks cortical granule exocytosis is probably by maintaining the intracytoplasmic level of ATP in aged oocytes.

In this study while rates of fertilization and blastocyst formation decreased significantly with time of oocyte aging without pyruvate supplementation, addition of pyruvate to aging medium increased both rates significantly. It is well known that pyruvate is an important chemical compound in biochemistry that provides energy for the maintenance of normal cellular activities. For example, under glucose-free conditions, exogenous

### Table 6: Intracellular glutathione (GSH) levels of mouse cumulus–oocyte complexes after aging for 12 h in CZB with or without pyruvate supplementation.

<table>
<thead>
<tr>
<th>Type of oocytes</th>
<th>Pyruvate (10 mM)</th>
<th>Total GSH</th>
<th>GSH</th>
<th>GSSG</th>
<th>GSH/GSSG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freshly ovulated</td>
<td>−</td>
<td>4.83±0.06(^a)</td>
<td>3.20±0.14(^a)</td>
<td>1.62±0.09(^a)</td>
<td>1.99±0.19(^a)</td>
</tr>
<tr>
<td>In vivo aging</td>
<td>−</td>
<td>3.90±0.10(^b)</td>
<td>1.25±0.08(^b)</td>
<td>2.70±0.08(^b)</td>
<td>0.45±0.03(^b)</td>
</tr>
<tr>
<td>In vitro aging</td>
<td>+</td>
<td>3.40±0.11(^c)</td>
<td>1.10±0.09(^c)</td>
<td>2.29±0.04(^c)</td>
<td>0.48±0.04(^c)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.18±0.15(^d)</td>
<td>1.95±0.04(^d)</td>
<td>2.23±0.14(^d)</td>
<td>0.88±0.06(^d)</td>
</tr>
</tbody>
</table>

\(^a–d\)Values with a common letter in superscripts did not differ significantly (P>0.05).

### Table 7: Ethanol-induced activation and maturation-promoting factor (MPF) activity after freshly ovulated cumulus-denuded oocytes (DOs) were cultured for 6 h in cumulus–oocyte complex (COC)- or DO-conditioned CZB with or without pyruvate supplementation.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Pyruvate (mM)</th>
<th>Oocytes observed</th>
<th>Percentage of oocytes activated</th>
<th>MPF activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CZB</td>
<td>0</td>
<td>108</td>
<td>12.3±1.7(^a,b)</td>
<td>92.2±3.2(^a)</td>
</tr>
<tr>
<td>DO-conditioned CZB</td>
<td>89</td>
<td>15.7±2.1(^b)</td>
<td>60.5±2.9(^b)</td>
<td>95.3±4.2(^b)</td>
</tr>
<tr>
<td>COC-conditioned CZB</td>
<td>110</td>
<td>63.4±3.2(^c)</td>
<td>75.9±2.8(^c)</td>
<td>98.7±3.4(^c)</td>
</tr>
<tr>
<td></td>
<td>0.27</td>
<td>110</td>
<td>35.4±1.7(^d)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>90</td>
<td>5.6±1.2(^e)</td>
<td></td>
</tr>
</tbody>
</table>

\(^a–d\)Values with a common letter in superscripts did not differ (P>0.05) in the same column.

*In each treatment, three samples collected on different experimental days were assayed for the relative activity of H1 kinase and each sample contained 40 oocytes.
Pyruvate doubled the growth of bovine pulmonary artery endothelial cells within 72 h (Chung et al. 2004). In addition, this study showed that pyruvate supplementation to aging medium increased the intracellular concentration of reduced GSH and also the level of the anti-apoptotic BCL2 proteins in aged mouse oocytes. Boerjan & de Boer (1990) also reported that the level of GSH was twice lower in mouse oocytes aged postovulation for 12 h than in unaged oocytes. It is known that cells generate reactive oxygen species (ROS) as byproducts through normal metabolic activities (Fridovich 1986, Yu 1994). The increased intracellular ROS has been suggested to induce DNA fragmentation, plasma membrane damage, and ultimately cell death (Buttke & Sandstrom 1994). Furthermore, ROS-induced apoptosis has also been observed in mammalian oocytes (Tatemoto et al. 2000, Chaube et al. 2003), mainly by inducing apoptosis in various types of somatic cells (Ramakrishnan et al. 1998, Kang et al. 2001, Mongan et al. 2002, Jagtap et al. 2003, Lee et al. 2003), mainly by maintaining the level of GSH (Mongan et al. 2002), increasing the ratio of anti-apoptotic molecules BCL2 or BCL2L1 to pro-apoptotic molecule BAX (Mongan et al. 2002, Lee et al. 2003) and by inhibiting the activation of caspase-3 (Mongan et al. 2002, Jagtap et al. 2003, Lee et al. 2004).

The present results showed that pyruvate supplementation inhibited acetylation of lysine 12 of histone H4 (H4K12) and lysine 14 of histone H3 (H3K14) in aged mouse oocytes. Huang et al. (2007) found that the acetylation levels of lysine 14 on histone H3 and lysines 8 and 12 on histone H4 increased gradually during in vivo and in vitro postovulatory aging of mouse oocytes. While histones were globally deacetylated in oocytes from young (3-week-old) mice (Kim et al. 2003), histones remained acetylated in the oocytes from old (10-month-old) mice (Akiyama et al. 2006). Inhibition of meiotic histone deacetylation has been found to induce aneuploidy in fertilized mouse oocytes, which resulted in embryonic death in utero at an early stage of development (Akiyama et al. 2006). Furthermore, it has been shown that treatment with inhibitors of histone deacetylase induced apoptosis of cancer cells and some of such inhibitors have been used as anticancer agents (Cheong et al. 2003, Maggio et al. 2004, Balasubramanian et al. 2008). In summary, it is possible that pyruvate prevents oocyte aging by providing energy for oocyte metabolism and by inhibiting apoptosis.

This study showed that more pyruvate was needed to prevent oocyte aging in the presence than in the absence of cumulus cells. Furthermore, when freshly ovulated DOs were cultured for 6 h in COC-conditioned CZB without additional pyruvate, activation rates increased while MPF activity decreased significantly compared with control oocytes cultured in unconditioned CZB. When cultured in conditioned CZB supplemented with 10 mM additional pyruvate, however, both activation rates and MPF activity remained close to those of control oocytes. Downs et al. (2002) reported that 5–6 times more pyruvate was consumed by COCs than by DOs during maturation in the absence of glucose. This strongly suggests that cumulus cells accelerate oocyte aging by consuming pyruvate in the culture medium. However, surprisingly, our capillary electrophoresis failed to show any decrease in the pyruvate concentration measured in the COC-conditioned CZB, but instead, it increased significantly with conditioning time compared with that in fresh CZB, although the concentration of lactate decreased with time. Dumollard et al. (2007) proposed the presence of discrete pools of pyruvate inside the oocyte: one from the culture medium, which is rapidly metabolized by the mitochondria, while a second pool derived from lactate is poorly used by the mitochondria. Such intracellular compartmentation of pyruvate pools has also been described in neuronal and glial cells (Cruz et al. 2001, Zwingmann et al. 2001). According to Dumollard et al. (2007), the lactate-derived pyruvate may be preferentially converted in the cytosol to alanine by alanine aminotransferase (ALT), because bovine oocytes contain strong ALT activities (Cetica et al. 2003) and bovine and porcine embryos produce large amounts of alanine (Gopichandran & Leese 2003, Humpherson et al. 2005). Therefore, the increased pyruvate concentration in COC-conditioned medium in this study might have resulted from accumulation of lactate-derived pyruvate, which could neither be used by oocytes for energy production nor be converted to alanine due to decreased ALT activities in aged cells.

Table 8 Pyruvate and lactate concentrations in CZB medium conditioned with different cells.

<table>
<thead>
<tr>
<th>Media</th>
<th>Pyruvate concentration (mM)</th>
<th>Lactate concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CZB</td>
<td>0.272 ± 0.009³</td>
<td>31.5 ± 0.14³</td>
</tr>
<tr>
<td>CZB conditioned with COCs for 6 h</td>
<td>0.314 ± 0.005³</td>
<td>30.3 ± 0.11³</td>
</tr>
<tr>
<td>CZB conditioned with COCs for 12 h</td>
<td>0.375 ± 0.024⁴</td>
<td>27.7 ± 1.21⁴</td>
</tr>
<tr>
<td>CZB conditioned with DOs for 6 h</td>
<td>0.276 ± 0.013³ ⁵ ⁶</td>
<td>30.9 ± 0.12⁴</td>
</tr>
<tr>
<td>CZB conditioned with DOs for 12 h</td>
<td>0.283 ± 0.010³ ⁵ ⁶</td>
<td>31.2 ± 0.25⁵ ⁶</td>
</tr>
</tbody>
</table>

³⁶Values with a common letter in their superscripts did not differ significantly (P>0.05) in the same column.
In conclusion, inhibiting oocyte aging is important not only for the healthy reproduction of humans and other mammals but also for the success of assisted reproduction techniques. Although our previous studies showed that cumulus cells accelerated aging of mouse oocytes, the exact mechanism by which cumulus cells promote oocyte aging is unknown. We have studied the effects of pyruvate and cumulus cells on mouse oocyte aging. The results showed that pyruvate inhibited aging of mouse oocytes, and reduced the detrimental effect of cumulus cells on oocyte aging. This suggests that the in vitro aging of oocytes results mainly from an insufficiency of energy or other substrates in the currently used culture medium. Thus, the current use of 0.27 mM pyruvate in the CZB medium was enough only for preventing aging of DOs while 5 mM or more pyruvate was needed for protecting the COCs. Furthermore, the amount for pyruvate supplementation will also depend upon the duration of oocyte culture. For example, with supplementation of 10 mM pyruvate, blastocyst rates following fertilization of aged oocytes decreased significantly from 6 to 12 h of in vitro aging. Our unpublished data also showed that the developmental potential of mouse oocytes aging in vitro for 12 h was better protected with 10 mM than with 5 mM pyruvate, although both concentrations inhibited oocyte activation at 6 h of culture. Together, the present study has provided important information that can be potentially used for the formulation of more optimal media in the clinical assisted reproduction technology.

Materials and Methods

Chemicals and reagents used in this study were purchased from Sigma Chemical Co. unless otherwise specified.

Oocyte recovery

Mice of the Kunming breed were kept in a room with 14 h light:10 h 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. Female mice, 6–8 weeks after birth, were induced to super-ovulate with equine chorionic gonadotropin (eCG, 10 IU, i.p.) followed 48 h later by human chorionic gonadotropin (hCG, 10 IU, i.p.). Both eCG and hCG used in this study were from Ningbo Hormone Product Co., Ltd, Cixi City, People’s Republic of China. The superovulated mice were killed at different times after hCG injection, and the oviductal ampullae were broken to release COCs at different stages of in vivo aging. After dispersed and washed thrice in M2 medium, some of the COCs were denuded of cumulus cells by pipetting with a thin pipette in a drop of M2 containing 0.1% hyaluronidase.

Preparation of conditioned medium

The CZB medium (200 μL, 0.27 mM pyruvate) was conditioned for 6 h with freshly ovulated COCs or DOs (30 per well) in wells of a 96-well culture plate. The conditioned medium was then aspirated from the wells and centrifuged at 300 g for 10 min to remove cells and debris, and frozen at −20 °C before being thawed for use.

In vitro aging of oocytes

For in vitro aging, COCs and DOs were cultured in the CZB medium (containing 0.27 mM pyruvate) supplemented with different concentrations of pyruvate. Some DOs were cultured in the COC-conditioned medium supplemented with or without 10 mM pyruvate to study the mechanism for conditioned medium to accelerate oocyte aging. The culture was conducted in wells (20–25 oocytes per well) of a 96-well culture plate containing 200 μL of medium, covered with mineral oil, at 37 °C under 5% CO2 in humidified air. At different time intervals of in vitro aging, the COCs and DOs were allocated to parthenogenetic activation, assay of MPF activity, in vitro insemination, or examination of other aging parameters.

Oocyte activation

Before treatment, COCs were denuded of cumulus cells by pipetting in M2 containing 0.1% hyaluronidase. Oocytes were first treated with 5% (v/v) ethanol in M2 medium for 5 min at room temperature, then washed thrice, and cultured in CZB (0.27 mM pyruvate) containing 2 mM 6-dimethylaminopurine (6-DMAP) for 6 h. At the end of the culture, oocytes were observed under a microscope for activation. Only those oocytes that had one or two pronuclei, or two cells each having a nucleus, were considered activated. Oocytes for controls were cultured for 6 h in CZB containing no 6-DMAP without prior ethanol treatment. Controls were set for each experiment and data were used only when no control oocytes were activated in the experiment.

Histone H1 kinase assay

Histone H1 kinase activity was measured as described previously (Wu et al. 1997) with modifications. In each treatment, three samples collected on three different experimental days were assayed and each sample contained 40 oocytes. Oocytes were denuded of cumulus cells if any before assay. Oocytes were washed thrice in the histone kinase buffer (15 mM 3-[N-morpholino] propanesulfonic acid, pH 7.2, containing 80 mM glycerophosphate, 10 mM EGTA, 15 mM MgCl2, 0.1 mM phenethylsulphonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 10 μg/ml cAMP-dependent protein kinase inhibitor peptide), transferred to 10 μl histone kinase buffer contained in a 1.5 ml microfuge tube and stored frozen at −70 °C. Prior to kinase reactions, the frozen samples were subjected to 4–5 times freezing and thawing to prepare lysates. Kinase reactions were initiated by the addition of 10 μl of substrate buffer containing 2 mg/ml histone H1, 2 mM dithiothreitol and 5 μCi [γ-32P] ATP to each sample, and the reactions were carried out for 50 min at 36 °C. The reaction was terminated by the addition of an equal volume of double-strength SDS sample buffer containing β-mercaptoethanol, and the mixture was boiled for 3 min. Kinase reaction products were then separated by 12% linear gradient SDS-PAGE.
Gels were exposed to Kodak X-ray film for 5 days at -20 °C for autoradiography. The autoradiographic image of the histone H1 bands was photographed using a digital camera and the band density was measured using the Adobe Photoshop 6.0 computer software. Six fields were measured in each band and an average value was calculated. The histone H1 activity values of freshly ovulated oocytes were arbitrarily set as 100%, and the other values were expressed relative to this activity. The amount of kinase reaction product used for SDS-PAGE was strictly controlled (20 μl) for each sample.

**IVF**

Masses of dense sperm were collected from the cauda epididymis of fertile male mice and were placed at the bottom of a test tube containing T6 medium supplemented with 10 mg/ml BSA. After 3–5 min, the supernatant containing highly motile spermatozoa were removed and capacitated in the same medium under mineral oil at 37 °C for 1.5 h. Oocytes were stripped of their cumulus cells if any by pipetting in M2 containing 0.1% hyaluronidase. To facilitate sperm penetration, a hole of about 20 μM in diameter was made on the ZP of some of the oocytes using a piezo-driven micromanipulator. After being washed in the fertilization medium (T6 containing 20 mg/ml BSA), the oocytes were placed in fertilization drops (20 oocytes per 40 μl drop). Capacitated sperm were added to the fertilization drops to give a final sperm concentration of about 1 X 10⁶/ml. After 6 h of incubation, oocytes were observed under a microscope for fertilization. Only those oocytes with two pronuclei and the second polar body were considered fertilized. The fertilized oocytes were cultured for 4 d in the regular CZB medium (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 embryos were cultured beyond the 3- or 4-cell stages.

**Assay for ZP hardening**

The assay for ZP hardening was performed as described by Liu et al. (2005). Briefly, 20 cumulus-free oocytes were treated with 1 mg/ml a-chymotrypsin (type II, 40–60 U/mg protein, C-4129) contained in a 100 ml drop of PBS covered with mineral oil. The treatment was conducted in a room with temperature maintained at 30 °C. Oocytes were monitored every 2 min during the first 30 min of treatment and then every 5 min until the end of the treatment. The time at which 75% of the ZP underwent a complete dissolution (with ooplasm stuck on the bottom of the dish) was assessed as t₅₀ for ZP dissolution.

**Immunofluorescence microscopy**

*Cortical granule staining*

The ZP was removed by treating oocytes with 0.5% pronase (Roche Diagnostics GmbH; Roche Diagnostic Corporation) in M2. After being washed thrice 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 thrice 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 for 5 min each in blocking solution. They were then cultured in 100 mg/ml of FITC-labeled lens culinaris agglutinin in M2 for 30 min in the dark. Finally, the oocytes were washed thrice in the washing solution and stained for 10 min with 10 μg/ml propidium iodine (PI) for chromatin examination.

**BCL2 staining**

The assay for BCL2 level was performed as described by Tatone et al. (2006) with modifications. Briefly, zona-free oocytes were fixed with 3.7% paraformaldehyde in M2 for 30 min at room temperature. The oocytes were then permeabilized for 5 min in 0.1% Triton X-100 in PBS-PVA, and washed in blocking buffer (PBS–PVA, 0.1% BSA, 0.01% Tween-20). Permeabilized oocytes were incubated with a rabbit anti-BCL2 polyclonal antibody (1:100; NovoGene Lifesciences Pvt Ltd, Mumbai, India) for 1 h at room temperature, followed by incubation with 1:200 dilution of a secondary goat anti-rabbit IgG conjugated with FITC (NovoGene). Finally, the oocytes were washed thrice in the blocking solution. In each experiment, negative control samples in which the primary antibody was omitted were also evaluated.

**Histone acetylation staining**

Oocytes were immunostained with antibodies against acetylated lysine 12 (Upstate Biotechnology Inc., Lake Placid, NY, USA) of histone H4 and lysine 14 (Upstate Biotechnology) of histone H3 as described by Kim et al. (2003) with slight modifications. In brief, zona-free oocytes were first fixed with 3.7% paraformaldehyde for 30 min and then permeabilized with 0.5% Triton X-100 for 15 min. The permeabilized oocytes were incubated with rabbit antibodies against the acetyl histones (1:100) for 1 h at room temperature or overnight at 4 °C. The antibodies that bound to the oocytes were probed with FITC-conjugated anti-rabbit IgG (1:100, Novogene) for 1 h at room temperature. Counterstaining was conducted with Hoechst 33342 to visualize the DNA.

**Laser confocal microscopy**

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

**Assay for intracellular glutathione**

Intracellular content of glutathione (GSH) was measured as described by Funahashi et al. (1994). Cumulus-free oocytes were washed thrice in M2. Five microliters of distilled water
containing 35–40 oocytes were 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 thrice. 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)–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 microliters 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 of 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 the total (GSSG+GSH) and the GSSG for each oocyte and expressed as pmol/oocyte.

Capillary electrophoresis

Capillary electrophoresis was performed on a P/ACE-MDQ system (Beckman Coulter, Fullerton, CA, USA). Three medium samples collected on different experimental days were analyzed for each treatment. The samples were analyzed in an electrolyte consisting of 100 mM disodium hydrogen phosphate and 0.5 mM cetyl trimethylammonium bromide (pH 7.0). The capillary used was with a total length of 60 cm, corresponding to a detection window of 48 cm and an inner diameter of 50 μm. Separation was performed with a sample injection from a minus pole at 3 p.s.i. at 20°C with an applied voltage of 10 kV. Analytes were detected by an excitation wavelength of 200 nm. Each sample was analyzed up to three times. The capillary was rinsed for 3 min by 1 M HCl, bidistilled water, 0.1 M sodium hydroxide and electrolyte respectively. Before run, between two samples, the capillary was rinsed for 2 min with electrolyte. Standard curves and peak positions to determine sodium pyruvate concentrations were generated using sodium pyruvate standards.

Data analysis

There were at least three replicates for each treatment. Percentage data were arc-sine transformed and analyzed with ANOVA; a Duncan multiple comparison test was used to locate differences. The software used was Statistics Package for Social Science (SPSS 11.5; SPSS Inc., Chicago, IL, USA). Data were expressed as mean ±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.

Funding

This study was supported by grants from the Momentous Basic Research Programs of the China Ministry of Science and Technology (No. 2006CB944003 and No. 2007CB947403) and the National Natural Science Foundation of China (No. 30771556, No. 30571337 and No. 30430530).

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Received 2 April 2009
First decision 5 May 2009
Accepted 21 May 2009