Effects of Sodium Pyruvate in Nonserum Maturation Medium on Maturation, Fertilization, and Subsequent Development of Bovine Oocytes With or Without Cumulus Cells

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ABSTRACT

The present study was conducted to determine the effects of cumulus cells and sodium pyruvate during in vitro maturation of bovine oocytes on maturation, fertilization, and subsequent development. Cumulus-enclosed oocytes (CEOs) and cumulus-denuded oocytes (CDOs) were cultured for 24 h in polyvinylpyrrolidone-Hepes-tissue culture medium 199 with or without sodium pyruvate. Oocytes were fertilized in vitro and then cultured in CR1aa for 10 days. Before in vitro fertilization, the glutathione (GSH) content of some oocytes was measured. Maturation and normal fertilization rates of CEOs cultured with sodium pyruvate and CEOs were higher than those of CDOs cultured without sodium pyruvate. The CEOs showed significantly higher rates of development to the blastocyst stage than CDOs. The GSH contents of oocytes significantly decreased in CDOs after maturation culture, but the GSH contents of oocytes in CEOs remained at the same level as oocytes before culture. These results indicate that sodium pyruvate promotes nuclear maturation of bovine CEOs and that a continuing presence of cumulus cells during maturation is important for subsequent development of zygotes to the blastocyst stage. However, blastocysts produced from CDOs in the presence of sodium pyruvate showed a developmental competence to be normal calves, but it is not known if CDOs cultured without sodium pyruvate also were capable of developing into calves.

cumulus cells, IVF/ART, meiosis, oocyte development

INTRODUCTION

Cumulus-oocyte complexes are maintained by delicate cell-to-cell connections among the cumulus cells and with the oocytes [1, 2]. Corona radiata cells, part of the cumulus cells surrounding oocyte, penetrate through the zona pellicuda and communicate with oocyte via the gap junctions [3]. These intracellular communications allow metabolite transfer [4] and play an important role during oocyte growth and maturation [5]. Isolated mouse cumulus cells and porcine cumulus-cell-oocyte complexes also formed pyruvate [6, 7]. It is generally accepted that cumulus cells during the maturation period support in vitro maturation (IVM) of oocytes to the metaphase-II stage and are involved in the cytoplasmic maturation needed for developmental competence of postfertilization such as male pronuclear formation in porcine oocytes [8]. However, there are reports that cumulus-denuded oocytes (CDOs) can complete meiotic maturation in mice [9], rat [10], sheep [11], pig [12], and cattle [13] in vitro. Although mature CDOs are not morphologically different from oocytes matured within the cumulus (CEOs) after completion of nuclear maturation, many reports indicate a lower developmental competence of CDOs after in vitro fertilization (IVF) than CEOs [11–15]. Recently, Yamauchi and Nagai [12] reported that the addition of cysteamine to culture medium increased oocyte glutathione (GSH) content and promoted male pronuclear formation after sperm penetration of porcine CDOs but had no effects on their maturation rates or kinase (histone H1 kinase and mitogen-activated protein kinase) activities. These poor developmental competencies of CDOs might reflect a difference in cytoplasmic maturation of oocytes. However, the functional role of cumulus cells during oocyte maturation on cytoplasmic maturation in cattle is still unknown.

Studies on cumulus cell function during IVM of oocytes were reported mainly by using serum containing maturation medium [13, 16]. The use of serum, however, in those experiments precluded an understanding of the functions of cumulus cells or chemical compounds in the process of oocyte maturation, because serum is undefined and contains many unknown constituents in varying concentrations, and the data interpretation is therefore obscured. For a full understanding of mechanism by which cumulus cells and/or a compound such as pyruvate exert effects on IVM of oocytes, the use of a chemically defined medium (without sera) is essential. And the understanding of the mechanism will lead to improve in vitro production of bovine embryos.

It has been reported that bovine CDOs can complete meiotic maturation in vitro and some denuded oocytes can develop to the 8- to 32-cell stage [13], morula, or blastocyst stage [17]. However, there are, to our knowledge, no published studies about the developmental competence to be calves of blastocysts obtained from bovine denuded immature oocytes matured in serum-free medium.

This study was conducted to determine the effects of cumulus cells and sodium pyruvate during IVM of bovine oocytes on their GSH contents, maturation, fertilization, and subsequent development in vitro by using serum-free culture systems.

MATERIALS AND METHODS

In Vitro Maturation

Unless specified, all reagents were from Sigma Chemicals (St. Louis, MO). Bovine ovaries were obtained from a slaughterhouse and were transported to the laboratory in sterile 0.9% NaCl solution at 35 to 39°C within 5 h of slaughter. Oocytes were aspirated from follicles (3 to 6 mm in diameter) with an 18-gauge needle attached to a disposable syringe. Oocytes covered with multilayers of cumulus cells (more than three cumulus layers) were selected and
separated into two groups, one in which the oocytes remained cumulus enclosed (CEOs) and the other in which the oocytes were denuded by mechanically pipetting, the CDOs group. After removing cumulus cells, oocytes with normal membrane, cytoplasm, and shape were selected. The base medium for maturation culture of oocytes was modified tissue culture medium 199 (mTCM-199), which is 25 mM Hapes-buffered TCM-199 (Gibco BRL, Grand Island, NY) containing 3 mg/ml polyvinylpyrrolidone (PVP) and 0.02 AU/ml porcine FSH (Denka Pharmaceutical, Kawasaki, Japan). Oocytes were then cultured at 38.5°C for 24 h in 50 μl of mTCM-199 with or without 0.2 mM sodium pyruvate (Wako Pure Chemical Industries, Osaka, Japan) under liquid paraffin (Nakarai Tesque, Kyoto, Japan) in an atmosphere of 5% CO₂ in air. Each drop of medium contained approximately 10 oocytes.

In Vitro Fertilization

Following culture, expanded cumulus-oocyte complexes were stripped free of cumulus cells by repeated pipetting in the presence of 0.1% hyaluronidase. Oocytes derived from CEOs and CDOs were transferred, for insemination, into modified Tyrode medium without glucose, supplemented with 6 mg/ml BSA, 1 μM epinephrine, 10 μM hypotaurine, 20 μM penicillinamine, and 5 μl/ml heparin (Shimizu Pharmaceutical, Shimizu, Japan) [18] under liquid paraffin. Insemination was achieved by adding a motile fraction of frozen-thawed bull sperm obtained by density gradient separation (Percoll) [19], with a final sperm concentration of 2 × 10⁶ cells/ml. Sperm and oocytes were coincubated for 6 h at 38.5°C in 5% CO₂ in air.

Culture of Embryos

After 6-h insemination, the inseminated oocytes were stripped free of any adherent sperm and transferred to 50-μl drops of the serum-free medium CR1aa [20] under liquid paraffin at 38.5°C in 5% CO₂, 5% O₂, and 90% N₂. Embryos were cultured in the medium for 5 days with the medium being replaced with an identical fresh medium containing 5.56 mM glucose (Wako Pure Chemical Industries) and then cultured for an additional 5 days. The initial development of embryos was evaluated at 54 h after insemination, and development of embryos to the blastocyst and hatching/hatched blastocyst stages were assessed at 8 days and 10 days after insemination. The experiment was repeated four times.

Assessment of Maturation and Fertilization Rates of Oocytes

In nine independent experiments, oocytes were evaluated at 24 h after maturation culture to assess maturation rate, and also, in eight independent experiments, oocytes were evaluated at 10 h after insemination to assess fertilization rates. The oocytes were fixed for 24 to 48 h with acetoethanol (acetic acid:ethanol, 1:3, v:v), stained with 1% ace-toorcein, and examined under phase-contrast microscope. Maturation to metaphase II, sperm penetration, and male and female pronuclear formation were assessed. Oocytes that had one set of male and female pronuclei and one sperm tail were considered to be fertilized normally.

Assay of GSH

Selected CEOs before maturation and CEOs and CDOs that were cultured in maturation medium with or without sodium pyruvate were used for the assay of GSH content. Following culture, expanded cumulus-oocyte complexes were stripped free of cumulus cells by repeated pipetting in the presence of hyaluronidase. All oocytes were washed five times in saline containing 3 mg/ml PVP. Groups of 50 (CEOs) or 100 (CDOs) oocytes in 5 μl of distilled water containing 3 mg/ml PVP were transferred to 1.5-ml microcentrifuge tubes, and 5 μl of 1.25 M H₃PO₄ was added. Then the samples were centrifuged at 2000 × g for 5 min and frozen at −80°C until assayed. The GSH content of oocytes was determined by the 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB)-GSSG reductase recycling assay according to Funahashi et al. [21]. Briefly, 700 μl of 0.33 mg NADPH/ml 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 of distilled water were added and mixed in a microcentrifuge tube. Ten microliters of 250 IU glutathione reductase/ml was added with mixing to initiate the reaction. The absorbance was monitored continuously at 412 nm with a spectrophotometer (Hitachi, Tokyo, Japan) for 3 min, with readings recorded every 0.5 min. The GSH standards (0.1 to 1.0 nmol) and a sample blank lacking GSH was also assayed. The amount of GSH was determined according to Calvin et al. [22].

Statistical Analysis

Statistical analyses were carried out by ANOVA with Fisher’s protected least-significant difference test using the Statview program (SAS Institute, Inc., Cary, NC). All percentage data were subjected to arc sine transformation before statistical analysis [23]. Data were expressed as mean ± SEM. A probability of P < 0.05 was considered to be significant statistically.

RESULTS

As shown in Table 1, the maturation rate of CDOs cultured without sodium pyruvate was significantly lower than those of the other three groups (P < 0.05). About half (44.6 ± 4.1%) of CDOs cultured without pyruvate were arrested at anaphase I, telophase I, and prometaphase II. There were no differences in penetration rates among treatments, but normal fertilization rates of CDOs cultured with sodium pyruvate and CEOs were higher than that of CDOs cultured without sodium pyruvate (Table 1). Although there was no difference in cleavage rates among treatments, embryos obtained from CEOs showed significantly higher rates of development to the four-cell (Day 2), blastocyst (Day 8), or hatching/hatched blastocyst (Day 10) stage than CDOs (P < 0.05) (Table 2). There was no difference in blastocyst formation rates between CDOs cultured with and without sodium pyruvate, but hatched blastocysts were obtained only in CDOs cultured with sodium pyruvate.

The GSH contents of oocytes before and after a 24-h maturation culture in medium with or without sodium pyruvate were shown in Figure 1. The GSH content of germinal vesicle (GV) stage oocytes (before culture) was 6.86 ± 0.19 pmol/oocyte. There were no differences among the GSH contents of CEOs and GV stage oocytes (before culture). But CDOs showed a significantly lower content of intercellular GSH than CEOs.

When two blastocysts (Day 7; Day 0 = insemination) produced from CDOs matured in serum-free medium supplemented with sodium pyruvate were transferred nonsurgically to synchronous two recipient cows (one blastocyst to each cow), both were pregnant and two normal male
cumulus cell complexes are capable of producing pyruvate cubated with glucose and lactate [6], and porcine oocyte-follicular cells are present in the culture medium [24]. Isoport their maturation, but glucose can do so only when subsequent development of zygotes to the blastocyst stage. of cumulus cells during maturation is important for subse-quent development of bovine CDOs and that a continuing presence serum maturation medium supports (promotes) nuclear maturation [25]. These findings suggest that cumulus cells maturation might affect further development of IVM/IVF em-bryos. Furthermore, the increased rates of normal fer-tilization in CDOs cultured with sodium pyruvate did not guarantee the subsequent development to the four-cell stage or the blastocyst stage, but hatching/hatched blastocysts were obtained from CDOs cultured with sodium pyruvate, not from CDOs cultured without sodium pyruvate. Sodium pyruvate during maturation of bovine oocytes might be nec-essary for hatching of blastocysts. Taken together, it was suggested that the existence of cumulus cells might affect the normal fertilization of bovine oocytes leading to normal development of the fertilized oocytes and/or the subsequent development of IVM/IVF em-bryos. Besides pyruvate, something in cumulus cells during maturation might affect further development of bovine IVM/IVF embryos. Further studies are needed to clarify this point.

Synthesis of GSH during oocyte maturation occurs in

calves were born at Day 286 (36 kg) and Day 293 (50 kg) of gestation. They grew up and the body weight at 120 days old was 111.9 kg and 119.6 kg, respectively. At the same time, one blastocyst produced from CEOs in the presence of sodium pyruvate was transferred to a recipient, but the recipient was not pregnant.

**DISCUSSION**

The present study shows that sodium pyruvate in non-serum maturation medium supports (promotes) nuclear maturation of bovine CDOs and that a continuing presence of cumulus cells during maturation is important for subse-quent development of zygotes to the blastocyst stage.

In mouse cumulus-free oocytes, pyruvate can also support their maturation, but glucose can do so only when follicular cells are present in the culture medium [24]. Isolated mouse cumulus cells also formed pyruvate when incubated with glucose and lactate [6], and porcine oocyte-cumulus cell complexes are capable of producing pyruvate to meet metabolic needs [7]. Pyruvate metabolism of bovine cumulus-free oocytes increased during IVM, while total glucose metabolism and the production of 14CO2 from [1,14C]glucose were low and relatively constant throughout maturation [25]. These findings suggest that cumulus cells metabolize glucose to pyruvate or Krebs cycle intermediates that can be passed to the oocyte and enhance its quality as shown by high maturation rates in all CEOs and CDOs cultured with sodium pyruvate in our study. Therefore, it was suggested that sodium pyruvate has a physiologically important role in IVM of bovine oocytes.

Research in cattle and other species also showed a ben-efficial effect of cumulus cells on IVM and IVF [13, 15, 26, 27]. Although some denuded oocytes can complete the mei-otic resumption, their capability of being fertilized normally and developing to normal embryos is significantly reduced as shown in the present study and by others in previous reports [11, 17]. Schroeder and Eppig [28] have shown that mouse oocytes, matured with cumulus cells, have greater fertilizability than those matured without cumulus cells. In contrast, bovine CDOs showed the same rates of penetration as CEOs in our study. However, bovine CDOs cultured without sodium pyruvate showed significantly lower rates of normal fertilization than CDOs cultured with sodium pyruvate and CEOs, mainly because of lower rates of maturation, in turn, causing lower rates of both male and female pronuclear formation after IVF in CDOs cultured without sodium pyruvate than CDOs cultured with sodium pyruvate and CEOs. Furthermore, the increased rates of normal fer-tilization in CDOs cultured with sodium pyruvate did not guarantee the subsequent development to the four-cell stage or the blastocyst stage, but hatching/hatched blastocysts were obtained from CDOs cultured with sodium pyruvate, not from CDOs cultured without sodium pyruvate. Sodium pyruvate during maturation of bovine oocytes might be nec-essary for hatching of blastocysts. Taken together, it was suggested that the existence of cumulus cells during oocyte maturation might affect the normal fertilization of bovine oocytes leading to normal development of the fertilized oocytes and/or the subsequent development of IVM/IVF em-bryos. Besides pyruvate, something in cumulus cells during maturation might affect further development of bovine IVM/IVF embryos. Further studies are needed to clarify this point.

Synthesis of GSH during oocyte maturation occurs in

### TABLE 1. Effects of cumulus cells and sodium pyruvate in the maturation medium on maturation and fertilization of bovine oocytes in vitro.

<table>
<thead>
<tr>
<th>Source of oocytes</th>
<th>With (+) or without (−) Na pyruvate (0.2 mM)</th>
<th>No. of trials</th>
<th>No. of oocytes examined</th>
<th>% Oocytes matureda</th>
<th>No. of trials</th>
<th>% Oocytes fertilized normallyd</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDOs</td>
<td>−</td>
<td>9</td>
<td>275</td>
<td>19.6 ± 6.6b</td>
<td>8</td>
<td>63.3 ± 6.8b</td>
</tr>
<tr>
<td>CEOs</td>
<td>−</td>
<td>9</td>
<td>229</td>
<td>77.7 ± 1.8b</td>
<td>8</td>
<td>79.5 ± 4.8b</td>
</tr>
<tr>
<td>CDOs</td>
<td>+</td>
<td>9</td>
<td>191</td>
<td>71.9 ± 5.7f</td>
<td>8</td>
<td>68.9 ± 7.9e</td>
</tr>
<tr>
<td>CEOs</td>
<td>+</td>
<td>9</td>
<td>216</td>
<td>78.5 ± 2.8f</td>
<td>8</td>
<td>79.6 ± 4.4d</td>
</tr>
</tbody>
</table>

aOocytes at metaphase II, fixed at 24 h after maturation culture in nine independent experiments.

bOocytes were fixed at 10 h after insemination in eight independent experiments.

cPercentage of penetrated oocytes (having one or more sperm in the ooplasm) calculated per examined oocytes.
dPercentage of normally fertilized oocytes (having one set of male and female pronuclei and a sperm tail in the ooplasm) calculated per examined oocytes.

### TABLE 2. Effects of cumulus cells and sodium pyruvate in the maturation medium on subsequent development of bovine oocytes in vitro after IVF.

<table>
<thead>
<tr>
<th>Source of oocytes</th>
<th>With (+) or without (−) Na pyruvate (0.2 mM)</th>
<th>No. of trials</th>
<th>No. of oocytes examined</th>
<th>% IVM-IVF oocytes developing to stage:</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDOs</td>
<td>−</td>
<td>4</td>
<td>147</td>
<td>Cleaveda: 77.0 ± 4.9b</td>
</tr>
<tr>
<td>CEOs</td>
<td>−</td>
<td>4</td>
<td>164</td>
<td>72.9 ± 8.7b</td>
</tr>
<tr>
<td>CDOs</td>
<td>+</td>
<td>4</td>
<td>156</td>
<td>70.4 ± 2.5b</td>
</tr>
<tr>
<td>CEOs</td>
<td>+</td>
<td>4</td>
<td>165</td>
<td>74.4 ± 2.7b</td>
</tr>
</tbody>
</table>

aEmbryos developing to the two-cell stage or more advanced stages, observed 54 h after in vitro insemination.
bEmbryos developing to the four-cell stage or more advanced stages, observed 54 h after in vitro insemination.
cEmbryos developing to the blastocyst stage or more advanced stages, observed 8 days after in vitro insemination.
dEmbryos developing to the hatching or hatched blastocyst stages, observed 10 days after in vitro insemination.

Values are expressed as mean percentages ± SEM, and values with different superscripts within the same column are significantly different (P < 0.05).
mice [22], hamsters [29], pigs [30], and cattle [31–33]. After penetration, GSH participates in sperm decondensation in parallel with oocyte activation, as well as in the transformation of the penetrated sperm head into the male pronucleus [22, 29, 34–36]. In addition, GSH is known to have important roles in maintaining the redox state of cells and protecting cells against harmful effects of oxidative injuries [37, 38]. However, the antioxidant capacity is not unlimited, and the pool of reduced thiols becomes rapidly exhausted while disulfide oxidation products accumulate [39]. Thus, the beneficial effects of low molecular weight thiol compounds on the development of bovine four- to eight-cell embryos to the blastocyst stage is reported to be related with an increase of intracellular GSH levels [40]. In addition, it has been reported [41] that coculture cells such as cumulus cells take up cystine and produce cysteine for synthesis of GSH in embryos, as well as promoting synthesis of thiol embryos can utilize. In our study, when GSH content of CEOs and CDOs after culture was measured, CEOs showed a significantly higher content of intercellular GSH than CDOs. Therefore, it was suggested that one of the cumulus cell’s functions during maturation is to reduce cystine to cysteine and to promote the uptake of cysteine in oocytes, resulting in stable levels of GSH in the matured oocytes and a subsequent increased number of embryos that fertilized normally and develop to the blastocyst stage.

In this study, normal calves were produced from blastocysts obtained from CDOs matured in serum-free medium supplemented with sodium pyruvate. This is, to our knowledge, the first report of calves obtained from embryos derived from denuded immature oocytes matured, fertilized, and cultured in vitro using serum-free medium. These results demonstrate that blastocysts obtained from CDOs matured in a serum-free medium supplemented with sodium pyruvate are developmentally competent to become calves, although the percentage of embryos that develop to the blastocyst stage is low. It is not known if CDOs cultured without sodium pyruvate also are capable of developing into calves.

It can be concluded that a continuing presence of cumulus cells during oocyte maturation is important for the subsequent development of a zygote to the blastocyst stage in cattle. We found that cumulus cells promote GSH synthesis in bovine CEOs and are essential for embryo development after IVF. Also, in the absence of cumulus cells, sodium pyruvate in the serum-free medium supports (promotes) nuclear maturation of bovine CDOs. But even though CDOs could be matured in vitro by addition of sodium pyruvate in the medium, the rates of IVM-IVF oocytes developed to the four-cell or blastocyst stage are low. Nevertheless, bovine CDOs matured in the serum-free medium with sodium pyruvate can give rise to calves. The use of this chemically defined culture system to investigate the functions of cumulus cells or the effects of several factors during maturation will be useful for understanding of their mechanisms involved in the process of oocyte maturation. Further studies are required to improve the developmental competence of bovine oocytes matured without cumulus cells.

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