Meiotic competence and DNA damage of porcine oocytes exposed to an elevated temperature

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Abstract

The present study was conducted to investigate the effects of the length of exposure to an elevated temperature (41 °C) on the meiotic competence and DNA damage of porcine oocytes. Oocytes were recovered from ovaries, loaded into straws, and then exposed at 41.0 or 38.5 °C (sham control) for 0, 0.5, 1.0, or 1.5 h, followed by culture for 44 h. The proportion of oocytes reaching the metaphase II (MII) stage gradually decreased with increasing exposure time, irrespective of the exposure temperature. A higher proportion of oocytes stored at 38.5 °C reached MII (57–63%) than those exposed to 41 °C (14–29%; P < 0.01). The proportion of total oocytes with DNA fragmentation gradually increased with increasing exposure time, irrespective of the exposure temperature. The proportion of DNA fragmentation in total oocytes exposed to 41 °C (37–57%) was higher (P < 0.01) than that in total oocytes stored at 38.5 °C (14–24%). When the oocytes were stored at 38.5 °C for up to 1.5 h, there were no differences in the proportions of MII-stage oocytes, with DNA-fragmented nuclei among all groups (P > 0.05). However, a higher proportion of MII-stage oocytes exposed to 41 °C for more than 1 h exhibited DNA-fragmented nuclei, compared with MII-stage oocytes stored at 38.5 °C (P < 0.05). In conclusion, exposure of porcine oocytes to an elevated temperature had a detrimental effect on the meiotic competence and quality of oocytes; furthermore, the effect was dependent on the duration of exposure.

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1. Introduction

In vitro-produced mammalian embryos are usually derived from oocytes collected from ovaries obtained at an abattoir. In general, the ovaries are preserved in physiological saline at approximately 30–35 °C and transported to the laboratory. During transportation of ovaries and manipulation of oocytes, the oocytes (within or outside ovarian follicles) are often exposed to a wide range of temperatures that may ultimately affect subsequent development. Cooling of mammalian oocytes to sub-physiological temperatures is well known to affect their viability through the induction of various abnormalities at all stages of meiosis [1–4]. In particular, porcine oocytes at the germinal vesicle (GV) stage were highly sensitivity to chilling [5]. In a previous study, we also confirmed that the meiotic competence of porcine oocytes from ovaries stored at less than 25 °C decreased [6]. However, little informa-
tion is available concerning the effects of elevated temperature during the manipulation of oocytes on oocyte quality and meiotic competence.

It is noteworthy that elevated temperature is a major factor responsible for the reduced fertility in farm animals during the hot season in tropical areas. Heat stress (HS) can compromise reproductive events by decreasing the expression of estrous behavior, altering ovarian follicular development, compromising oocyte competence, and inhibiting embryonic development [7,8]. Based on field fertility data, conception rates in cattle were significantly lower during the hot summer compared with the cool season [9]. Moreover, embryo viability in cows was lower in the hot season than in the cool season [10]. In most mammalian species, the deleterious effects of HS on embryonic mortality were most pronounced at or near the time of estrous, relative to the rest of the estrous cycle [11–13].

Direct exposure of bovine oocytes at the GV-stage to an elevated temperature (41 °C) for 12 h reduced their ability to complete nuclear maturation and development after fertilization [14]. Moreover, some studies have demonstrated DNA fragmentation and cytoskeleton disruption of oocytes after direct exposure to elevated temperature before or during maturation culture [15,16]. Roth and Hansen [15] reported that elevated temperatures within the physiological range (40–41 °C) during maturation culture increased programmed cell death in bovine oocytes. These observations suggested that the exposure of oocytes to elevated temperature induced DNA damage in oocytes prior to fertilization.

The objective of this study was to investigate the effects of the length of exposure to an elevated temperature (41 °C) on the meiotic competence and DNA damage of GV-stage porcine oocytes.

2. Materials and methods

2.1. Exposure to an elevated temperature and in vitro maturation (IVM) of oocytes

Ovaries from prepubertal crossbred gilts, approximately 6 months old, were collected at a local abattoir and transported to the laboratory (within 3 h) in physiological saline [0.85% (w/v) NaCl] at 35 °C. The cortex of each ovary was sliced repeatedly with a scalpel blade to release cumulus–oocyte complexes (COCs) from antral follicles in a 90-mm culture dish containing modified-PBS (mPBS; Embryotech, Nihon Zenyaku Kogyo, Fukushima, Japan). The COCs with at least two dense layers of cumulus cells were collected and washed twice with mPBS. To assess the effects of exposure of oocytes to elevated temperature on oocyte meiotic competence and nuclear damage, the COCs were randomly assigned to seven groups. The COCs were transferred into fresh mPBS and then loaded into 0.25-mL French plastic straws (I.M.V., L’Aigle, France), with 7 or 10 COCs per straw. The plastic straws were loaded as follows: a 5 cm length of the straw was filled with mPBS, followed by a 0.5 cm air bubble, approximately 1.0 cm of mPBS containing the COCs, another air bubble, the mPBS (approximately 3 cm) and a further air bubble. The straws were stored at 41 °C in an incubator for 0.5, 1.0, or 1.5 h. In order to evaluate the exposure conditions (sham control), some straws containing the COCs were stored at 38.5 °C for 0, 0.5, 1.0, or 1.5 h. After each exposure time, the contents of the straws were drained into polystyrene culture dishes. The COCs were immediately transferred into a maturation medium, a modified North Carolina State University (NCSU)-37 solution [17] supplemented with 0.6 mM cysteine, 1 mM dibutyryl cyclic AMP (dbcAMP; Sigma, St. Louis, MO, USA), 10 IU/mL equine chorionic gonadotropin (eCG; KawasakiMitaka K.K., Tokyo, Japan), 10 IU/mL human chorionic gonadotropin (hCG; KawasakiMitaka K.K.), 50 μg/mL gentamicin (Sigma) and 10% (v/v) porcine follicular fluid. Approximately 10 COCs were cultured (for 22 h) in each 100 μL drop of the maturation medium, covered with a layer of mineral oil (Sigma) in a 35 mm × 10 mm Petri dish. They were then transferred to the maturation medium without hormones and dbcAMP and cultured for an additional 22 h. All cultures were performed in a 38.5 °C humidified incubator containing 5% CO2 in air.

2.2. Analysis of meiotic stage and DNA damage of oocytes

After maturation culture, the meiotic stage and DNA damage of oocytes were analyzed using a combined technique for simultaneous nuclear staining and the terminal deoxynucleotidyl transferase (TdT) nick-end labeling (TUNEL), by a modification of the procedures previously described by Otoi et al. [18]. Briefly, oocytes were mechanically denuded from cumulus cells in PBS (Invitrogen, Carlsbad, CA, USA), supplemented with 1 mg/mL hyaluronidase (Sigma). Denuded oocytes were washed four times in PBS containing 3 mg/mL polyvinylalcohol (PBS–PVA) and fixed overnight at 4 °C in 3.7% (v/v) paraformaldehyde diluted in PBS. After fixation, the oocytes were washed four times in PBS–PVA, permeabilized in PBS containing 0.1% (v/v) Triton-X100 for 1 h, and incubated in a PBS containing...
10 mg/mL BSA (blocking solution) overnight at 4 °C. They were then washed four times in PBS–PVA and incubated in fluorescein-conjugated dUTP and TdT (TUNEL reagent; Roche Diagnostics, Tokyo, Japan) for 1 h at 38.5 °C under 5% CO2 in air. Positive controls (1 or 2 oocytes per TUNEL analysis) were incubated in 1000 IU/mL deoxyribonuclease I (DNase; Sigma) for 30 min at 38.5 °C under 5% CO2 in air and washed twice in PBS–PVA before TUNEL staining. Negative controls (1 or 2 oocytes per TUNEL analysis) were incubated in fluorescein-dUTP in the absence of TdT. After TUNEL staining, the oocytes were washed three times in PBS–PVA and counterstained with 25 μg/mL bis-benzimid (Hoechst 33342; Sigma) for 30 min. They were then washed in blocking solution, treated with an anti-bleaching solution (Slow-Fade; Molecular Probes, Eugene, OR, USA), mounted on a glass slide, and sealed with clear nail polish. Labeled oocytes were examined using a Nikon Diaphot microscope fitted with epifluorescence illumination. Two standard filter sets were used, a filter with an excitation wavelength of 450–490 nm and a barrier filter of 520 nm were used to detect fluorescein isothiocyanate (FITC) alone. A filter with an excitation wavelength of 450–490 nm and a barrier filter of 420 nm were used to detect the nuclear status of oocytes stained with Hoechst 33342. Oocytes that had been cultured for 44 h were observed to ascertain if they had reached metaphase II (MII) stage. In order to assess DNA damage in oocytes after maturation culture, the numbers of nuclei labeled by TUNEL were counted (Fig. 1).

### 2.3. Statistical analysis

The data are expressed as mean ± S.E.M. The proportions of oocytes reaching the MII-stage, and oocytes with DNA-fragmented nuclei were subjected to arcsin transformation prior to ANOVA, followed by the post hoc Fisher’s protected least significant difference test (PLSD test) using the Statview program (Abacus Concepts, Inc., Berkeley, CA, USA). For all analyses, \( P < 0.05 \) was considered significant.

### 3. Results

The proportions of oocyte nuclear maturation and DNA fragmentation of oocytes following exposure of GV-stage oocytes to 38.5 °C or to an elevated temperature (41 °C) are shown in Table 1. When the oocytes were stored at 38.5 °C for up to 1.5 h, the proportion of oocytes reaching MII gradually decreased with increasing storage time. Similarly, the proportion of DNA fragmentation in the total oocytes examined at the end of IVM culture increased with increasing storage time. However, there were no differences in the proportions of MII-stage oocytes with DNA-fragmented nuclei among all the groups exposed to 38.5 °C \( (P > 0.05) \). A higher proportion of oocytes stored at 38.5 °C reached MII (57–63%) than those oocytes exposed to 41 °C (14–29%), irrespective of the duration of treatment \( (P < 0.01) \).

The proportion of oocytes reaching MII after exposure to 41 °C decreased with increasing exposure time. In addition, the proportions of DNA fragmentation in the total and MII-stage oocytes exposed to 41 °C increased with increasing exposure time. When the oocytes were exposed to 41 °C for 1 h or more, the proportions of MII-stage oocytes with DNA-fragmented nuclei were higher \( (P < 0.05) \) than when stored at 38.5 °C. Moreover, the proportions of DNA fragmentation in total oocytes exposed to 41 °C were higher \( (P < 0.01) \) than in those stored at 38.5 °C, irrespective of the treatment times.

### 4. Discussion

In the present study, exposure of porcine oocytes to an elevated temperature (41 °C) clearly caused a reduction in their maturation rate and increased the proportion of oocytes with DNA-fragmented nuclei. In contrast, a previous study demonstrated that when in vitro-matured bovine oocytes were exposed to 40.5–41.5 °C for 1 h, heat stress had no deleterious effects on the developmental competence of oocytes [19]. Payton et al. [14] reported that the ability of bovine oocytes to develop into blastocysts after in vitro maturation and fertilization was not compromised, even by direct exposure of the GV-stage oocytes to high temperature (41 °C) for 6 h before maturation culture. However, in the present study, the meiotic competence of porcine oocytes exposed to 41 °C decreased, even after exposure for only 0.5 h. Tong et al. [20] reported that although exposure of porcine ovaries to 41.3–42.1 °C for 30 min during slaughter did not compromise the nuclear maturation of oocytes, it induced extensive disruption of oocyte cytoskeletal organization that decreased the developmental competence of parthenogenetically activated oocytes. Porcine oocytes/embryos are more sensitive to low temperatures compared with those of other mammalian species [21,22]. This heightened sensitivity may be related to their relatively high lipid content and/or lipid composition [23]. In that regard, porcine oocytes have a twofold greater complement of fatty acids than bovine and ovine...
In the present study, when oocytes were stored in mPBS at 38.5 °C, the proportion of DNA fragmentation in total oocytes examined at the end of IVM culture gradually increased. However, the proportion of DNA fragmentation in MII-stage oocytes did not differ among the treatment groups. Therefore, when the oocytes were stored in mPBS, the storage time affected oocytes, reflecting the acyl-containing lipid mass [24]. Although a direct comparison of the present study with previous studies is difficult due to differences in species, temperatures and the duration of exposure to high temperatures [14,19], we inferred that porcine oocytes may be more sensitive, than oocytes from other species, to both low and high temperatures.

Fig. 1. Representative DNA fragmentation in porcine GV-stage (A, a, B and b, 200 ×) and MII-stage (C, c, D and d, 200 ×) oocytes stained with Hoechst 33342 (left) and terminal deoxynucleotidyl transferase (TdT) nick-end labeling (TUNEL; right). GV- and MII-stage oocytes with (a and c, respectively) and without (b and d, respectively) DNA-fragmented nuclei.
Conversely, when the oocytes were exposed to 41°C, the quality of oocytes that reached the MII-stage decreased. The quality of total oocytes, but that it does not influence the quality of oocytes that reached the MII-stage. Moreover, the proportions of DNA fragmentation in total oocytes exposed to 41°C were higher than in those stored at 38.5°C, irrespective of the duration of treatment. The effects of exposure of oocytes to 41°C on the quality of oocytes were dependent on the exposure time. Ju and Tseng [16] reported that abnormalities in the chromosomes, spindle microtubules and pericytoplasmic microtubules of porcine oocytes occurred when the oocytes were exposed to 41.5°C for a short interval (1 h). Furthermore, they suggested that these deleterious effects of hyperthermia on porcine oocytes were irreversible, even if the oocytes were returned to normal culture conditions. It has been demonstrated that mouse GV-stage oocytes exposed to 43°C reduced synthesis of intracellular proteins and that the heat-induced reduction of protein synthesis intensified as the duration of the heat shock increased [25]. Heat shock during oocyte maturation promoted an apoptotic response mediated by group II caspases, which are responsible for the destruction of structural and regulatory proteins that lead to DNA damage and cell demise [15,26]. Roth and Hansen [15] suggested that activation of apoptotic processes mediated by the group II caspases, caused by heat shock during oocyte maturation, was a critical mechanism responsible for the disruption of oocyte capacity for cleavage and subsequent development. Conversely, cumulus cell function not only supported the oocyte maturation associated with developmental competence, but also served as scavengers, removing toxic materials from the culture medium [27]; cumulus cells played a critical role in the protection of the oocyte against apoptosis-inducing oxidative stress, through the enhancement of glutathione content in the oocyte [28]. Although the mechanism by which elevated temperature-induced oocyte DNA damage remains unclear, increased activity of group II caspases and disruption of the interactions between the oocyte and its cumulus investments by heat shock may be responsible for the induction of oocyte apoptosis.

In summary, exposure of porcine oocytes to an elevated temperature (41°C) for a short interval (1 h) decreased the meiotic competence of oocytes and had a detrimental effect on the quality of oocytes; furthermore, the effect was dependent on the duration of exposure.

Table 1
Mean (±S.E.M.) meiotic maturation and DNA fragmentation of porcine IVM oocytes following exposure to 38.5 or 41°C for varying intervals

<table>
<thead>
<tr>
<th>Exposure temperature (°C)</th>
<th>Duration of exposure (h)</th>
<th>No. of oocytes examined</th>
<th>No. of MII oocytes (%)</th>
<th>No. of MII oocytes with DNA-fragmented nuclei (%)</th>
<th>No. of total oocytes with DNA-fragmented nuclei (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>38.5</td>
<td>0</td>
<td>121</td>
<td>81 (66.7 ± 1.6) a</td>
<td>4 (4.8 ± 2.1) a</td>
<td>10 (7.4 ± 2.0) a</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>111</td>
<td>70 (63.1 ± 1.9) a,b</td>
<td>4 (5.8 ± 2.6) a</td>
<td>19 (14.1 ± 2.4) a,b</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>115</td>
<td>70 (61.1 ± 1.5) b,c</td>
<td>4 (9.9 ± 2.8) a</td>
<td>29 (21.0 ± 3.8) b,c</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>114</td>
<td>65 (57.4 ± 2.4) c</td>
<td>6 (8.1 ± 2.6) a</td>
<td>32 (23.7 ± 3.3) c</td>
</tr>
<tr>
<td>41</td>
<td>0.5</td>
<td>119</td>
<td>34 (28.6 ± 1.9) d</td>
<td>6 (20.2 ± 7.8) a,b</td>
<td>54 (36.8 ± 2.9) d</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>111</td>
<td>19 (17.1 ± 2.1) e</td>
<td>6 (32.1 ± 11.3) b,c</td>
<td>56 (43.3 ± 3.8) d</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>114</td>
<td>16 (14.0 ± 1.1) e</td>
<td>8 (50.0 ± 12.8) c</td>
<td>72 (56.8 ± 3.6) e</td>
</tr>
</tbody>
</table>

Within a column, values without a common letter differed (P < 0.05).

a Fourteen replicated trials were carried out.
b Oocytes collected from ovaries were transferred into straws and exposed to 38.5°C (sham treatment) or 41°C for various intervals.
c MII, metaphase II.
d Percentages were calculated by dividing the number of MII oocytes with DNA-fragmented nuclei by the total number of MII oocytes.

References


