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3 **SENSITIVITY OF THE MEIOTIC STAGE TO HYPERTHERMIA DURING *IN***
4 ***VITRO* MATURATION OF PORCINE OOCYTES**

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6 Katsutoshi NISHIO¹, Mado YAMAZAKI², Masayasu TANIGUCHI², Kazuhiko BESSHI³,
7 Fumio MORITA³, Toshiki KUNIHARA¹, Fuminori TANIHARA¹, Tatsuya TAKEMOTO⁴
8 and Takeshige OTOI^{1*}

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10 ¹Faculty of Bioscience and Bioindustry, Tokushima University, Tokushima 779-3233, Japan;11 ²Joint Faculty of Veterinary Science, Yamaguchi University, Yamaguchi, Japan; ³Drug Safety12 Research Laboratory, Taiho Pharmaceutical Co. Ltd., Tokushima, Japan; ⁴Fujii Memorial

13 Institute of Medical Sciences, Tokushima University, Tokushima, Japan

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15 **Running title:** SENSITIVITY OF MEIOTIC STAGE TO HYPERTHERMIA IN PIG
16 OOCYTE

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18 The present study was conducted to clarify the meiotic stage of porcine oocytes having
19 the highest sensitivity to hyperthermia during *in vitro* maturation by evaluating the meiotic
20 competence and DNA damage. Oocytes were exposed to 41 °C for 12 h at various intervals
21 during 48 h of maturation culture. When the oocytes were exposed to 41 °C from 12 to 24 h of
22 the maturation culture, the proportion of oocytes reaching metaphase II (MII) decreased as
23 compared to the control oocytes cultured at 38.5 °C ($P < 0.05$). Moreover, the proportions of
24 DNA fragmentation in all oocytes exposed to 41 °C in each culture period after 12 h from the
25 start of maturation culture were significantly higher ($P < 0.05$) than for the control oocytes.
26 When the meiotic stage of oocytes cultured at 38.5 °C between 12 and 24 h was examined, the
27 majority of oocytes remained at the germinal vesicle (GV) stage at 12 h and approximately
28 half of the oocytes reached metaphase I (MI) at 24 h. These results indicate that the meiotic

*Corresponding author; E-mail: otoi@tokushima-u.ac.jp; Phone/Fax: 0081-88-635-0963

29 stage of porcine oocytes having the highest sensitivity to hyperthermia during *in vitro*
30 maturation is a transition period from the GV stage to the MI stage.

31 **Key words:** Heat stress, maturation, meiotic stage, quality, sensitivity

32

33 **Introduction**

34

35 Heat stress (HS) can compromise reproductive events by decreasing the expression of
36 oestrous behaviour, altering follicular development, compromising oocyte competence, and
37 inhibiting embryonic development (Wolfenson et al., 2000; Hansen et al., 2001). HS disrupts
38 the synthesis of the steroid hormone involved in the regulating mechanism of oocyte
39 maturation. Moreover, HS during maturation has been suggested to alter both nuclear and
40 cytoskeletal configurations in oocytes, reduce developmental competence, and increase oocyte
41 apoptosis (Ju and Tseng, 2004; Roth and Hansen, 2004). In a previous study, we demonstrated
42 that the exposure of porcine oocytes at the germinal vesicle stage to an elevated temperature
43 (41 °C) causes a reduction in their maturation rate and increases the proportion of oocytes
44 with DNA-fragmented nuclei. Hypothermia-mediated DNA damage to the cumulus cells
45 surrounding the oocyte during maturation reduces the porcine oocyte quality, resulting in
46 failure of meiotic maturation (Yuan et al., 2008). The deleterious effects of hyperthermia on
47 porcine oocytes are potentially irreversible, even if the oocytes are returned to normal culture
48 conditions (Ju and Tseng, 2004). However, the meiotic stage of oocytes, during maturation,
49 with the most sensitivity to hyperthermia remains unclear.

50 The objective of this study was to clarify the meiotic stage of porcine oocytes that has the
51 most sensitivity to hyperthermia by assessing the meiotic maturation and DNA damage of
52 oocytes exposed to an elevated temperature (41 °C) for 12 h at various intervals during
53 maturation culture.

54

55 **Materials and methods**

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57 ***Exposure to an elevated temperature and in vitro maturation (IVM) of oocytes***

58 Porcine ovaries were obtained from prepubertal cross-bred gilts (Landrace, Large White
59 and Duroc breeds) at a slaughterhouse for an April-June 2014 and transported to the
60 laboratory within 3 h in physiological saline (0.9% (w/v) NaCl) at 30 °C. The ovaries were

61 washed three times with modified phosphate-buffered saline (m-PBS; Nihonzenyaku,
62 Fukushima, Japan) that was supplemented with 100 IU/ml penicillin G potassium (Meiji,
63 Tokyo, Japan) and 0.1 mg/ml streptomycin sulfate (Meiji). The cumulus–oocyte complexes
64 (COCs) were collected from 3-6-mm follicles using a surgical blade. Only COCs with a
65 uniform, dark-pigmented ooplasm and an intact cumulus cell mass were collected.
66 Approximately 50 COCs were then cultured in 500 µl of maturation medium consisting of
67 25 mM HEPES tissue culture medium 199 with Earle's salts (TCM 199; Invitrogen Co.,
68 Carlsbad, CA, USA) that was supplemented with 10% (v/v) porcine follicular fluid, 0.6 mM
69 cysteine (Sigma-Aldrich, St. Louis, MO, USA), 50 µM sodium pyruvate (Sigma-Aldrich), 2
70 mg/ml D-sorbitol (Wako Pure Chemical Industries Ltd., Osaka, Japan), 1 µg/ml 17 β-estradiol
71 (Sigma-Aldrich), 10 IU/ml equine chorionic gonadotropin (Kyoritu Seiyaku, Tokyo, Japan),
72 10 IU/ml human chorionic gonadotropin (Kyoritu Seiyaku), and 50 µg/ml gentamicin (Sigma-
73 Aldrich) for 24 h in 4-well dishes (Nunc A/S, Roskilde, Denmark). Subsequently, the COCs
74 were transferred to maturation medium without hormone supplementation and cultured for an
75 additional 24 h according to the method previously described by Namula et al. (2013).

76

77 *Analysis of the meiotic stage and DNA damage of oocytes*

78 After maturation culture, the meiotic stage and DNA damage of oocytes were analysed
79 with a combined technique for simultaneous nuclear staining and terminal deoxynucleotidyl
80 transferase (TdT) nick-end labelling (TUNEL) by a modification of the procedures previously
81 described by Otoi et al. (1999). Briefly, oocytes were mechanically denuded from cumulus
82 cells in Dulbecco's PBS (DPBS; Invitrogen Co) that was supplemented with 1 mg/mL
83 hyaluronidase (Sigma). Denuded oocytes were fixed overnight at 4°C in 3.7% (w/v)
84 paraformaldehyde diluted in DPBS. After fixation, the oocytes were permeabilized in DPBS
85 containing 0.1% (v/v) Triton-X100 for 40 min. They were subsequently incubated overnight at
86 4°C in DPBS containing 10 mg/ml bovine serum albumin (A9647, Sigma-Aldrich). The
87 oocytes were then incubated in fluorescein-conjugated 2'-deoxyuridine-5'-triphosphate and
88 terminal deoxynucleotidyl transferase (TUNEL reagent; Roche Diagnostics, Tokyo, Japan) for
89 1 h at 38.5°C. After TUNEL staining, the oocytes were counterstained with 1 µg/ml DAPI
90 (Invitrogen Co.) for 10 min. Then, they were treated with an anti-bleaching solution (Slow-
91 Fade; Molecular Probes Inc., Eugene, OR, USA), mounted on a glass slide, and sealed with
92 clear nail polish. Labelled oocytes were examined using a microscope (Eclipse 80i, Nikon,

93 Tokyo, Japan) with epifluorescence illumination. They were classified according to chromatin
94 configuration as being in the germinal vesicle (GV), condensed chromatin (CC), metaphase I
95 (MI), anaphase I to telophase I (AT), or metaphase II (MII) stage. Those with diffusely stained
96 cytoplasm characteristics of nonviable cells and those in which chromatin were unidentifiable
97 or not visible were excluded from DNA damage analysis.

98 To assess the meiotic stage of oocytes cultured at 38.5 °C for each period, oocytes were
99 fixed and permeabilized in DPBS containing 3.7% (w/v) paraformaldehyde and 1% (v/v)
100 Triton X-100 (Sigma-Aldrich) at room temperature for 15 min. They were then incubated in
101 DPBS containing 0.3% (w/v) polyvinylpyrrolidone at room temperature for another 15 min.
102 The oocytes were placed in a drop of mounting medium consisting of 90% (v/v) glycerol with
103 1.9 µM Hoechst 33342 (Sigma-Aldrich) on a slide, covered with a cover slip supported by
104 four droplets of Vaseline/paraffin, incubated overnight at 4 °C and examined under a
105 fluorescence microscope. The meiotic stage of oocytes was classified as described above.

106

107 ***Experiment 1***

108 To assess the sensitivity of the porcine oocyte meiotic stage to hyperthermia, the COCs
109 were randomly assigned to five treatment groups and then cultured in maturation medium at
110 41 °C for 12 h in each period during maturation culture. The COC incubations were
111 performed in a 38.5 °C humidified incubator containing 5% CO₂ with an exposure period of
112 41 °C. After 48 h of IVM culture, the oocytes were fixed and stained to examine the nuclear
113 status and DNA damage of oocytes exposed to 41 °C.

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115 ***Experiment 2***

116 In Experiment 1, the sensitivity of porcine oocytes exposed to hyperthermia from 12 h to
117 24 h after the start of maturation culture was higher than the other exposed groups. Therefore,
118 the meiotic stages of oocytes cultured in a 38.5 °C humidified incubator containing 5% CO₂
119 for each period during 48 h of maturation culture and between 12 and 24 h were examined.

120

121 ***Statistical analysis***

122 The data are expressed as the means ± SEMs. The proportions of oocytes reaching each
123 stage and oocytes with DNA-fragmented nuclei were subjected to arc sin transformation
124 before performing an analysis of variance (ANOVA). The transformed data were tested by

125 ANOVA, which was followed by the post hoc Fisher's protected least significant difference
126 test (PLSD test) using the Statview program (Abacus Concepts, Inc., Berkeley, CA, USA).
127 Differences at a probability value (P) of 0.05 or less were considered significant.

128

129 **Results**

130

131 ***Experiment 1***

132 As shown in Table 1, when the oocytes were exposed to 41 °C from 12 to 24 h, the
133 proportions of oocytes that remained at MI increased and of oocytes reaching MII decreased
134 compared with control oocytes cultured at 38.5 °C ($P < 0.05$). Moreover, the proportions of
135 DNA fragmentation in the total oocytes exposed to 41 °C at each culture period after 12 h
136 from the start of maturation culture were significantly higher ($P < 0.05$) than those of control
137 oocytes (Fig. 1). The proportions of MII-stage oocytes with DNA-fragmented nuclei tended to
138 be higher in oocytes exposed to 41 °C after 12 h of maturation culture than the control oocytes
139 ($P < 0.1$).

140

141 ***Experiment 2***

142 As shown in Fig. 2A, when the meiotic stages of the oocytes were examined at various
143 intervals during maturation culture, the proportions of oocytes remaining at the GV stage
144 dramatically decreased from 74.8% to 22.5% between 12 and 24 h after the start of maturation
145 culture. The proportions of oocytes at the CC and MI stages increased at 24 h of maturation
146 culture. The proportion (47.5%) of CC-stage oocytes reached a maximum at 20 h, and
147 approximately half (44.9%) of the oocytes reached MI at 24 h (Fig. 2B).

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149 **Discussion**

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151 Our previous study demonstrated that when porcine oocytes were exposed to 41.0 °C for
152 the entire period of maturation culture, their meiotic competence decreased, but the oocytes
153 could mature and develop to the blastocyst stage after fertilization (Do et al., 2015). In the
154 present study, porcine oocytes were exposed to 41.0 °C for 12 h at each period of maturation
155 culture to clarify the meiotic stage of porcine oocytes that had the most sensitivity to
156 hyperthermia. We confirmed that the exposure of porcine oocytes to 41 °C for 12 h decreased

157 the meiotic competence of oocytes and increased the DNA damage of total and MII-stage
158 oocytes. Moreover, porcine oocytes cultured from 12 to 24 h after the start of maturation
159 culture had a higher sensitivity to the elevated temperature.

160 The cooling of mammalian oocytes to sub-physiological temperatures is well known to
161 affect their viability through inducing various abnormalities at all stages of meiosis (Moor and
162 Crosby, 1985; Heyman et al., 1986; Pickering et al., 1990; Aman and Parks, 1994). In particular,
163 porcine oocytes at the GV stage have been demonstrated to have a high sensitivity to chilling
164 (Didion et al., 1990). Similarly, heat stress during porcine oocyte maturation has been shown to
165 retard the nuclear maturation of oocytes, resulting in the poor oocyte quality and low potency of
166 their development (Tseng et al., 2006; Yuan et al., 2008). Our previous study demonstrated that
167 exposure of porcine oocytes at the GV stage to 41 °C for 1 h reduced their maturation rate and
168 increased the proportion of oocytes with DNA-fragmented nuclei (Barati et al., 2008). Yuan et
169 al. (2008) also reported that the maturation rates of oocytes at the germinal vesicle breakdown
170 (GVBD) stage decreased with exposure to 42 °C for 1 h. It has been suggested that
171 abnormalities in the chromosomes, spindle microtubules, and pericytoplasmic microtubules of
172 porcine oocytes occurred when the oocytes were exposed to an elevated temperature for even a
173 short time (Ju and Tseng, 2004). Moreover, heat shock during oocyte maturation has been
174 shown to promote an apoptotic response that is mediated by group II caspases, which are
175 responsible for destruction of structural and regulatory proteins that leads to DNA damage and
176 cell demise (Chang and Yang, 2000; Roth and Hansen, 2004). Activation of the apoptotic
177 processes mediated by the group II caspases is a critical mechanism that is responsible for
178 disrupting the oocyte capacity to cleave and further develop (Roth and Hansen, 2004).
179 Although the detrimental effects of heat shock on the meiotic competence and quality of
180 oocytes has been demonstrated, the meiotic stage of oocytes with high sensitivity against
181 hyperthermia has remained unclear. In the current paper, we clearly showed that the detrimental
182 effects of hyperthermia become more apparent for the maturation rates and DNA damage of
183 oocytes that were exposed to 41.0 °C between 12 and 24 h after the start of maturation culture.
184 At that time, oocytes resumed meiosis from the GV stage, and the majority of the oocytes
185 reached the CC- or MI-stage after 24 h in culture. In the detailed analysis of the meiotic stage of
186 oocytes between 12 and 24 h in culture, GVBD started in the majority of oocytes after 20 h in
187 culture. These results were similar to the experiment by Nobata et al. (2013), who reported that
188 porcine oocytes remained at the GV stage after 12 h of maturation culture and GVBD started

189 after 18 h. Moreover, we observed that the proportion of oocytes at the GVBD stage reached
190 maximum at 20 h, and approximately half of oocytes reached the MI stage at 24 h. Therefore,
191 our results indicate that the transition period to the MI stage from the GV stage has higher
192 sensitivity to the elevated temperature.

193 In summary, the results of the present study demonstrate that porcine oocytes cultured from
194 12 to 24 h after the start of maturation culture had a higher sensitivity to hyperthermia, and their
195 meiotic stages were from the GV to MI stage.

196

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204 **References**

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283 **Figure legends**

284

285 **Figure 1**

286 Effects of porcine oocyte exposure to 41 °C for 12 h during maturation culture on the
287 proportions of total (A) and metaphase II (B) oocytes with DNA-fragmented nuclei. Control
288 oocytes were cultured for 48 h without exposure to 41 °C. Proportions were calculated by
289 dividing the number of oocytes with DNA-fragmented nuclei by the total number of oocytes
290 examined and metaphase II oocytes. Each bar represents the mean \pm SEM. Bars with different
291 letters differ significantly (a-c; $P < 0.05$, A-C; $P < 0.1$).

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293

294 **Figure 2**

295 Meiotic stage of porcine oocytes cultured for each time period during 48 h (A) and
296 between 12 and 24 h (B) of maturation culture. All oocytes cultured at 38.5 °C for each time
297 period during 48 h (116 -120 oocytes) and between 12 and 24 h (98 - 99 oocytes) were used to
298 estimate the meiotic stage.

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Table 1. Meiotic maturation of porcine oocytes exposed to 41 °C during in vitro maturation*

Exposure period	No. of oocytes examined	No. (%) of oocytes with**					No. (%) of unidentifiable oocytes
		GV	CC	MI	AT	MII	
Control	133	3 (2.0 ± 2.0)	8 (6.0 ± 3.2)	20 (15.1 ± 4.1) ^a	2 (1.5 ± 1.0)	95 (72.2 ± 3.6) ^a	5 (3.3 ± 1.9) ^{a,b}
0 h – 12 h	129	2 (1.8 ± 1.8)	4 (3.2 ± 1.5)	41 (30.8 ± 7.8) ^b	4 (3.4 ± 2.2)	76 (59.3 ± 7.1) ^{a,b}	2 (1.5 ± 1.0) ^a
12 h – 24 h	128	5 (4.3 ± 2.0)	5 (3.6 ± 1.3)	38 (28.8 ± 2.7) ^b	5 (4.1 ± 2.1)	66 (51.7 ± 4.3) ^b	9 (7.6 ± 2.9) ^b
24 h – 36 h	132	2 (1.3 ± 1.3)	9 (6.2 ± 2.2)	24 (19.1 ± 3.1) ^{a,b}	2 (2.0 ± 2.0)	86 (64.8 ± 2.2) ^{a,b}	9 (6.7 ± 2.2) ^{a,b}
36 h – 48 h	134	4 (3.2 ± 1.6)	3 (2.0 ± 1.4)	28 (20.9 ± 2.9) ^{a,b}	3 (2.5 ± 1.7)	92 (68.4 ± 4.2) ^a	4 (3.0 ± 1.6) ^{a,b}

*All experiments were repeated 6 times. Data are expressed as the mean ± SEM.

**GV, germinal vesicle; CC, condensed chromatin; MI, metaphase I; AT, anaphase I to telophase I; and MII, metaphase II.

^{a-b} The values with different superscript letters in the same column are significantly different ($P < 0.05$).

Fig. 1



