AVH 80/2014 Received 3 August 2016

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

Katsutoshi NISHIO[1], Mado YAMAZAKI[2], Masayasu TANIGUCHI[2], Kazuhiko BESSHI[3], Fumio MORITA[3], Toshiki KUNIHARA[1], Fuminori TANIHARA[1], Tatsuya TAKEMOTO[4] and Takeshige OTOI[1,*]

[1]Faculty of Bioscience and Bioindustry, Tokushima University, Tokushima 779-3233, Japan; [2]Joint Faculty of Veterinary Science, Yamaguchi University, Yamaguchi, Japan; [3]Drug Safety Research Laboratory, Taiho Pharmaceutical Co. Ltd., Tokushima, Japan; [4]Fujii Memorial Institute of Medical Sciences, Tokushima University, Tokushima, Japan

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

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

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

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

Introduction

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

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

Materials and methods

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

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

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

Analysis of the meiotic stage and DNA damage of oocytes

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

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

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

Experiment 1

To assess the sensitivity of the porcine oocyte meiotic stage to hyperthermia, the COCs were randomly assigned to five treatment groups and then cultured in maturation medium at 41 °C for 12 h in each period during maturation culture. The COC incubations were performed in a 38.5 °C humidified incubator containing 5% CO_2 with an exposure period of 41 °C. After 48 h of IVM culture, the oocytes were fixed and stained to examine the nuclear status and DNA damage of oocytes exposed to 41 °C.

Experiment 2

In Experiment 1, the sensitivity of porcine oocytes exposed to hyperthermia from 12 h to 24 h after the start of maturation culture was higher than the other exposed groups. Therefore, the meiotic stages of oocytes cultured in a 38.5 °C humidified incubator containing 5% CO_2 for each period during 48 h of maturation culture and between 12 and 24 h were examined.

Statistical analysis

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

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

Results

Experiment 1

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

Experiment 2

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

Discussion

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

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

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

Acknowledgments

We thank the Meat Inspection Office of Kitakyushu city (Japan) for supplying the porcine ovaries. This work was supported in part by Japan Science and Technology Agency/Japan International Cooperation Agency, Science and Technology Research Partnership for Sustainable Development (JST/JICA, SATREPS).

References

Aman, R. R. and Parks, J. E. (1994): Effects of cooling and rewarming on the meiotic spindle and chromosomes of in vitro-matured bovine oocytes. Biol. Reprod. **50**, 103-110.

Barati, F., Agung, B., Wongsrikeao, P., Taniguchi, M., Nagai, T. and Otoi, T. (2008): Meiotic competence and DNA damage of porcine oocytes exposed to an elevated temperature. Theriogenology **69**, 767-772.

Chang, H. Y. and Yang, X. (2000): Proteases for cell suicide: functions and regulation of caspases. Microbiol. Mol. Biol. Rev. **64**, 821-846.

Didion, B. A., Pomp, D., Martin, M. J., Homanics, G. E. and Markert, C. L. (1990): Observations on the cooling and cryopreservation of pig oocytes at the germinal vesicle stage. J. Anim. Sci. **68**, 2803-2810.

Do, L. T., Luu, V. V., Morita, Y., Taniguchi, M., Nii, M., Peter, A. T. and Otoi, T. (2015): Astaxanthin present in the maturation medium reduces negative effects of heat shock on the developmental competence of porcine oocytes. Reprod. Biol. **15**, 86-93.

219 Hansen, P. J., Drost, M., Rivera, R. M., Paula-Lopes, F. F., al-Katanani, Y. M., Krininger, C.
220 E., 3rd and Chase, C. C., Jr. (2001): Adverse impact of heat stress on embryo
221 production: causes and strategies for mitigation. Theriogenology **55**, 91-103.
222 Heyman, Y., Smorag, Z., Katska, L. and Vincent, C. (1986): Influence of carbohydrates,
223 cooling and rapid freezing on viability of bovine non-matured oocytes or 1-cell
224 fertilized eggs. Cryo-Letters **7**, 170-183.
225 Ju, J. C. and Tseng, J. K. (2004): Nuclear and cytoskeletal alterations of in vitro matured
226 porcine oocytes under hyperthermia. Mol. Reprod. Dev. **68**, 125-133.
227 Moor, R. and Crosby, I. (1985): Temperature-induced abnormalities in sheep oocytes during
228 maturation. J. Reprod. Fertil. **75**, 467-473.
229 Namula, Z., Sato, Y., Kodama, R., Morinaga, K., Luu, V. V., Taniguchi, M., Nakai, M.,
230 Tanihara, F., Kikuchi, K., Nagai, T. and Otoi, T. (2013): Motility and fertility of boar
231 semen after liquid preservation at 5 degrees C for more than 2 weeks. Anim. Sci. J. **84**,
232 600-606.
233 Nobata, T., Kyougoku, H. and Miyano, T. (2013): Hematoxylin staining reveals a decrease in
234 nucleolar diameter of pig oocytes before germinal vesicle breakdown. J. Reprod. Dev.
235 **59**, 500-505.
236 Otoi, T., Yamamoto, K., Horikita, N., Tachikawa, S. and Suzuki, T. (1999): Relationship
237 between dead cells and DNA fragmentation in bovine embryos produced in vitro and
238 stored at 4 degrees C. Mol. Reprod. Dev. **54**, 342-347.
239 Pickering, S. J., Braude, P. R., Johnson, M. H., Cant, A. and Currie, J. (1990): Transient
240 cooling to room temperature can cause irreversible disruption of the meiotic spindle in
241 the human oocyte. Fertil. Steril. **54**, 102-108.
242 Roth, Z. and Hansen, P. J. (2004): Involvement of apoptosis in disruption of developmental
243 competence of bovine oocytes by heat shock during maturation. Biol. Reprod. **71**,
244 1898-1906.
245 Tseng, J. K., Tang, P. C. and Ju, J. C. (2006): In vitro thermal stress induces apoptosis and
246 reduces development of porcine parthenotes. Theriogenology **66**, 1073-1082.
247 Wolfenson, D., Roth, Z. and Meidan, R. (2000): Impaired reproduction in heat-stressed cattle:
248 basic and applied aspects. Anim. Reprod. Sci. **60-61**, 535-547.
249 Yuan, Y., Hao, Z. D., Liu, J., Wu, Y., Yang, L., Liu, G. S., Tian, J. H., Zhu, S. E. and Zeng, S.
250 M. (2008): Heat shock at the germinal vesicle breakdown stage induces apoptosis in

surrounding cumulus cells and reduces maturation rates of porcine oocytes in vitro. Theriogenology **70**, 168-178.

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 ± SEM. Bars with different
291 letters differ significantly (a-c; $P < 0.05$, A-C; $P < 0.1$).

292

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.

299

300

301

302

303

304

305

306

307

308

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

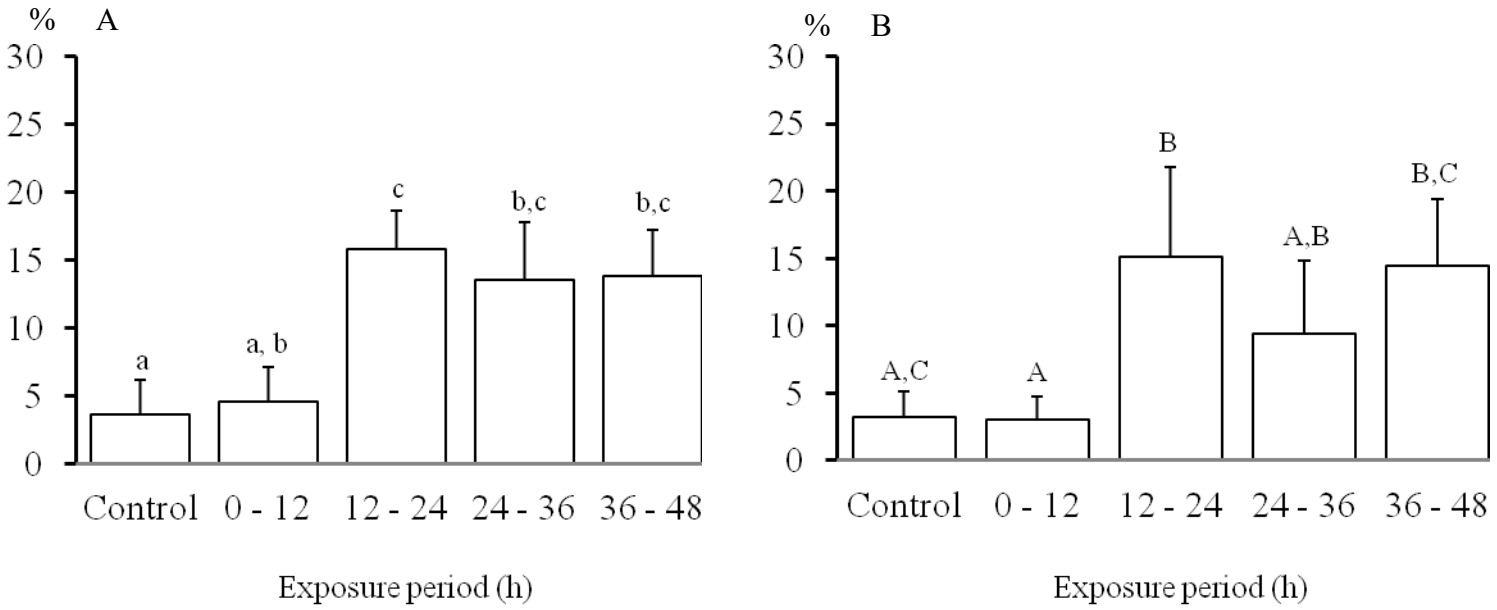
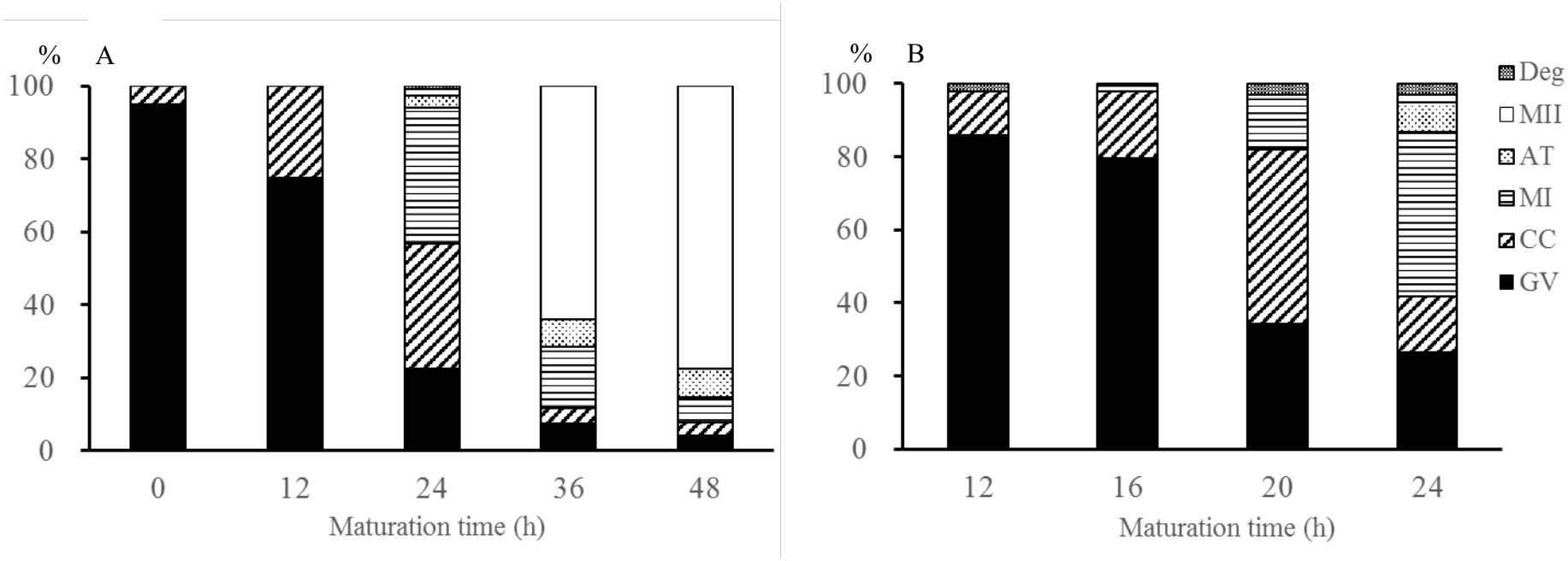

% A
30
25
20
15
10
5
0
a
a, b
c
b,c
b,c
Control
0 - 12
12 - 24
24 - 36
36 - 48
Exposure period (h)
% B
30
25
20
15
10
5
0
A,C
A
B
A,B
B,C
Control
0 - 12
12 - 24
24 - 36
36 - 48
Exposure period (h)

Fig. 2


%
A
100
80
60
40
20
0
0
12
24
36
48
Maturation time (h)
%
B
100
80
60
40
20
0
12
16
20
24
Maturation time (h)
Deg
MII
AT
MI
CC
GV