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Meiotic Competence and DNA Damage of Porcine Oocytes from Ovaries Exposed to an Elevated Temperature

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Abstract: The present study was conducted to investigate the effects of the exposure length of ovaries to an elevated temperature (41°C) on the meiotic competence and DNA damage of oocytes. Ovaries were stored in physiological saline at 41°C for 0 h (control), 0.5, 1.0 and 1.5 h. After exposure of ovaries to the elevated temperature, oocytes were collected and then cultured for 44 h. The length of exposure of ovaries to 41°C had no effect on the proportions of total oocytes with DNA-fragmented nuclei before maturation culture, but it did influence the proportions at the end of maturation culture. The proportion of oocytes reaching metaphase II (MII) significantly decreased with increasing exposure time. In addition, significantly more oocytes from ovaries exposed to 41°C for 1.5 h had DNA-fragmented nuclei compared with control oocytes. These results indicate that the meiotic competence and DNA damage of porcine oocytes are dependent on the duration of exposure of ovaries to the elevated temperature. Moreover, the occurrence of DNA damage in oocytes becomes more apparent after maturation culture than before the culture.

Key words: Heat stress, storage, apoptosis, oocyte maturation

INTRODUCTION

In vitro produced mammalian embryos are mostly derived from oocytes collected from ovaries obtained at the slaughterhouse. In general, the ovaries are preserved in physiological saline at approximately, 30-35°C and transported to the laboratory. During the collection and transportation of ovaries, the ovaries are often exposed to a wide range of temperatures that may ultimately affect subsequent development. The cooling of mammalian oocytes to sub-physiological temperatures is well known to affect their viability through the induction of various abnormalities (Moor and Crosby, 1985; Heyman *et al*., 1986; Pickering *et al*., 1990; Aman and Parks, 1994). In particular, porcine oocytes at the Germinal Vesicle (GV) stage have been demonstrated to have a high sensitivity to chilling (Didion *et al*., 1990).

In a previous study, we also confirmed that the meiotic competence of porcine oocytes from ovaries stored at less than 25°C decreased (Yuge *et al*., 2003). On the other hand, it has been reported that exposure of porcine ovaries to 41.3-42.1°C for 30 min during the slaughter process induced extensive disruption of oocyte cytoskeletal organization, resulting in a decrease in the developmental competence of parthenogenetically activated oocytes (Tong *et al*., 2004). Ju and Tseng (2004) also demonstrated that abnormalities in the chromosomes, spindle microtubules and pericytoplasmic microtubules of porcine oocytes occurred when the oocytes were directly exposed to 41.5°C for a short time (1 h). Furthermore, they suggested that these deleterious effects of hyperthermia on porcine oocytes are irreversible, even if the oocytes are returned to normal culture conditions. These observations suggest that the exposure of ovaries to elevated temperature decreases the meiotic competence of oocytes within follicles. However, little information is available concerning the relationships between the exposure length of ovaries to physical elevated temperature and the quality of oocytes within ovarian follicles.

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

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MATERIALS AND METHODS

Exposure of ovaries to an elevated temperature and *in vitro* maturation (IVM) of oocytes: Ovaries from prepubertal crossbred gilts, approximately 6 mo old, were collected at a local slaughterhouse and transported to the laboratory in physiological saline (0.85% (w v^{-1}) NaCl) at 35°C within 3 h. To assess the effects of the exposure of ovaries to an elevated temperature on the meiotic competence and nuclear damage of oocytes, a total of 80 ovaries were randomly assigned to 4 groups and then stored in physiological saline at 41°C for 0 h (control), 0.5, 1.0 and 1.5 h. After each storage time, the cortex of each ovary was sliced repeatedly with a scalpel blade to release the Cumulus-Oocyte Complexes (COCs) from antral follicles in a 90-mm culture dish containing modified phosphate-buffered saline (mPBS; Embryotech, Nihon Zenyaku Kogyo, Fukushima, Japan). Only COCs with 2 or more dense layers of cumulus cells were collected and then washed twice with mPBS. The COCs were transferred into a maturation medium, a modified North Carolina State University (NCSU) -37 solution (Petters and Wells, 1993) supplemented with 0.6 mM cysteine, 1 mM dibutyryl cyclic AMP (dbcAMP; Sigma, St. Louis, MO, USA), 10 IU mL^{-1} equine chorionic gonadotropin (eCG; KawasakiMitaka K.K., Kanagawa, Japan), 10 IU mL^{-1} human chorionic gonadotropin (hCG; KawasakiMitaka K. K.), 50 μg mL^{-1} gentamicin (Sigma) and 10% (v v^{-1}) porcine follicular fluid. Approximately, 10 COCs were cultured in each 100 μL drop of the maturation medium covered with a layer of mineral oil (Sigma) in 35×10 mm Petri dish for 22 h. 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% CO_2 in air.

Analysis of meiotic stage and DNA damage of oocytes: 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 the procedures previously described by Barati *et al.* (2008). Briefly, denuded oocytes were washed 4 times in PBS (Invitrogen, Carlsbad, CA, USA) containing 3 mg mL^{-1} polyvinylalcohol (PBS-PVA) and fixed overnight at 4°C in 3.7% (w v^{-1}) paraformaldehyde diluted in PBS. After fixation, the oocytes were permeabilized in PBS containing 0.1% (v v^{-1}) Triton -X100 for 1 h and incubated in a PBS containing 10 mg mL^{-1} bovine serum albumin (blocking solution) overnight at 4°C. They were then incubated in fluorescein-conjugated dUTP and TdT (TUNEL reagent; Roche Diagnostics, Tokyo, Japan) for 1 h at 38.5°C. Positive controls (1 or 2 oocytes per TUNEL analysis) were incubated in 1000 IU mL^{-1} deoxyribonuclease I (DNase; Sigma) for 30 min at 38.5°C 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 counterstained with 25 μg mL^{-1} bis-benzimide (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. Labeled oocytes were examined using a Nikon Diaphot microscope fitted with epifluorescence illumination. To assess the relationship between nuclear status and DNA damage, they were classified according to chromatin configuration as Germinal Vesicle (GV), Condensed Chromatin (CC), Metaphase I (MI) or Metaphase II (MII). Those with diffusely stained cytoplasm characteristics of nonviable cells and those in which chromatin were unidentifiable or not visible, were excluded from analysis of DNA damage.

Statistical analysis: The data are expressed as means±SEMs. The proportions of oocytes reaching each meiotic 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 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

The proportions of nuclear maturation and DNA fragmentation of oocytes derived from ovaries that were exposed to an elevated temperature (41°C) are presented in Table 1. When the duration of exposure of ovaries to the elevated temperature was prolonged, the proportion of oocytes reaching MII decreased from 59-16% ($p<0.05$). In contrast, once the oocytes reached the MII stage, there were no significant differences in the proportions of oocytes with DNA-fragmented nuclei between the oocytes from unexposed ovaries (control) and those exposed to the elevated temperature for up to 1.0 h. However, when the ovaries were exposed for 1.5 h, the MII-stage oocytes exhibited a higher proportion of DNA-fragmented nuclei than the MII-stage oocytes in the control ($p<0.05$). Similarly, the proportion of DNA fragmentation of the GV-MI stage oocytes from ovaries exposed for 1.5 h was significantly higher than that of the GV-MI stage oocytes from control ovaries ($p<0.05$).

Table 1: Meiotic maturation and DNA fragmentation of porcine IVM oocytes from ovaries exposed to 41°C for various times[†]

Exposure time (h)	No. of oocytes examined	No. (%) of oocytes[††] with		No. (%)[†††] of DNA-fragmented oocytes with	
		GV-MI	MII	GV-MI	MII
0	198	74 (36.9±5.0)[a]	116 (59.1±5.0)[a]	18 (26.3±7.8)[a]	11 (10.5±2.8)[a]
0.5	193	94 (50.7±6.9)[a,b]	67 (38.4±9.1)[b]	38 (37.5±9.2)[a, b]	5 (10.9±3.8)[a]
1.0	190	127 (66.8±2.4)[b]	43 (24.6±3.9)[b,c]	71 (54.2±7.1)[a, b]	11 (28.2±12.3)[a, b]
1.5	199	131 (65.2±6.5)[b]	26 (15.8±3.5)[c]	90 (59.5±10.5)[b]	12 (46.6±16.2)[b]

[†]Data are expressed as the mean±SEM. Five replicate trials were carried out; [††]GV-MI, germinal vesicle to metaphase I, MII, metaphase II; [†††]Percentages were calculated by dividing the number of oocytes with DNA-fragmented nuclei by the total number of oocytes reached each meiotic stage; [a-c]Values with different superscripts in the same column are significantly different ($p<0.05$)

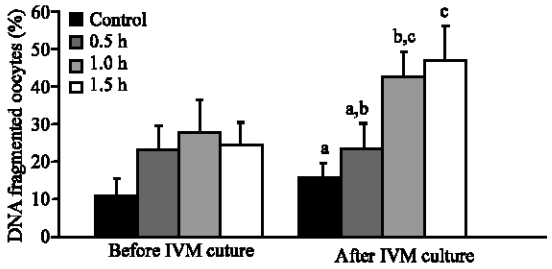

Fig. 1: Effects of exposure length of ovaries to 41°C on the proportions of total oocytes with DNA-fragmented nuclei before and after maturation culture. Control oocytes were collected from ovaries without exposure to 41°C. Proportions were calculated by dividing the number of oocytes with DNA-fragmented nuclei by the total number of oocytes examined before (n = 98~110) and after IVM (n = 190~199). Five replicate trials were carried out. Each bar represents the mean±SEM. Bars with different letters within each IVM culture differ significantly ($p<0.05$)

The proportions of DNA fragmentation in total oocytes examined at the onset and end of IVM culture after exposure of ovaries to the elevated temperature are shown in Fig. 1. There were no significant differences in the proportions of oocytes with DNA-fragmented nuclei among the treatment groups at the onset of IVM culture. At the end of IVM culture, however, the proportion of oocytes with DNA-fragmented nuclei increased with increasing time of exposure to the elevated temperature.

DISCUSSION

A previous study demonstrated that the ability of bovine oocytes to develop into blastocysts after *in vitro* maturation and fertilization is not compromised, even by direct exposure of the GV-stage oocytes to high temperature (41°C) for 6 h before maturation culture (Payton *et al*., 2004).

In the present study, however, the meiotic competence of porcine oocytes from ovaries exposed to 41°C, even for only 0.5 h, decreased. Moreover, its effect was dependent on the exposure time. It has been demonstrated that porcine oocytes/embryos are more sensitive to low temperatures compared with those of other mammalian species (Pollard and Leibo, 1994; Leibo *et al*., 1996). This heightened sensitivity has been suggested to be related to their relatively high lipid content and/or lipid composition (Nagashima *et al*., 1994). In fact, porcine oocytes have a twofold greater complement of fatty acids, reflecting the acyl-containing lipid mass, compared with bovine and ovine oocytes (McEvoy *et al*., 2000).

In a previous study, we found that when the GV-stage oocytes were directly exposed to 41°C for various times (0.5-1.5 h), the proportion of oocytes reaching MII after IVM culture decreased with increasing exposure time (Barati *et al*., 2008). Taken together, these results indicate that, compared with other species, porcine oocytes may exhibit a high sensitivity not only to low temperature but also to elevated temperature.

There have been many reports on apoptosis in cumulus and granulosa cells during oocyte maturation; these have demonstrated that the apoptosis of cumulus cells increases as the maturation of oocytes proceeds (Mikkelsen *et al*., 2001; Ikeda *et al*., 2003; Zeuner *et al*., 2003). However, there are very few reports concerning DNA damage in the oocyte itself before and after maturation culture.

In the present study, we found that the exposure length of ovaries to physical high temperature had no effect on the proportion of DNA fragmentation of total examined oocytes at the onset of IVM culture, but that it did influence the proportion at the end of IVM culture. The proportions of DNA fragmentation of both the GV-MI and MII stage oocytes from ovaries exposed for 1.5 h were significantly higher than those from control ovaries. 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 (Curci *et al*., 1987). Heat shock during oocyte maturation has been shown to promote 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 (Chang and Yang, 2000; Roth and Hansen, 2004). Roth and Hansen (2004) suggested that activation of apoptotic processes mediated by the group II caspases, which is caused by heat shock during oocyte maturation, is a critical mechanism responsible for the disruption of oocyte capacity for the cleavage and subsequent development. On the other hand, prolonged storage of ovaries has been shown to influence cumulus morphology and to increase the apoptosis of granulosa cells (Pedersen *et al*., 2004). Cumulus cells function not only supports the oocyte maturation associated with developmental competence but also acts as scavengers, removing toxic materials from the culture medium (Khurana and Niemann, 2000). The cumulus cells play a critical role in the protection of the oocyte against apoptosis-inducing oxidative stress through, the enhancement of the glutathione content in the oocyte (Tatemoto *et al*., 2000). Therefore, an increased activity of group II caspases and disruption of the interactions between the oocyte and its cumulus investments by the exposure of ovaries to the elevated temperature might induce oocyte apoptosis and increase the proportions of oocytes with DNA fragmentation after IVM culture.

In the present study, ovaries were transported to the laboratory in physiological saline at 35°C within 3 h and then exposed to physical high temperature for 0-1.5 h. During transportation of ovaries to the laboratory, the occlusion of blood flow reduces the supply of oxygen and energy to the ovaries and places them under ischemic and re-oxygenation conditions. The transportation of ovaries without blood supply may affect the oocyte quality by influencing the extracellular environment surrounding the oocytes. Moreover, the exposure of the ovaries to high temperature may accelerate deterioration in follicular environments. In a previous study, we found that a long-term storage induce acidosis of follicular fluid by ischemia condition of ovary, leading DNA fragmentation of oocytes in follicles (Wongsrikeao *et al*., 2005). Therefore, the transportation of ovaries at 35°C before the exposure to an elevated temperature might accelerate the effect of the exposure length on DNA damage of oocytes.

CONCLUSION

In summary, the results of the present study demonstrate that exposure of porcine ovaries to an elevated temperature (41°C) for a short time (0.5 h) decreased the meiotic competence of oocytes and its effect was dependent on the exposure time. Moreover, the occurrence of DNA damage of oocytes became more apparent after IVM culture and the DNA damage of MII-stage oocytes increased when the ovaries were exposed to the elevated temperature for 1.5 h.

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