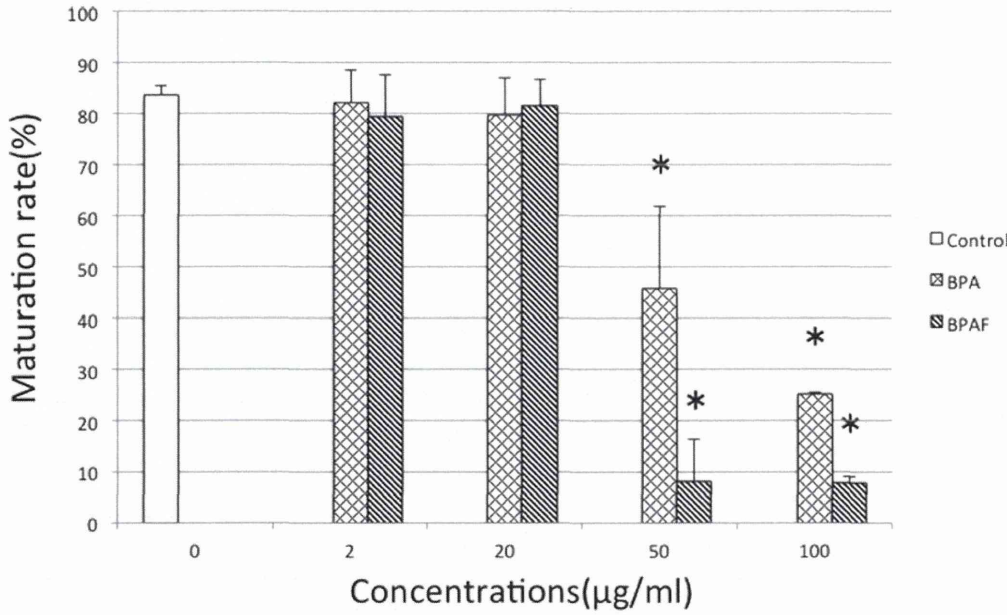

Figure 1 Maturation rate of oocytes exposed to BPA or BPAF at different concentrations. Oocytes were cultured for 18 h. Values are the means ± standard error of the mean (SEM) of three replicates. Bars with different letters are significantly different (*$P < 0.05$).

clarify whether oocytes progress to metaphase II (MII) after BPA or BPAF exposure, COCs were cultured for 12 h in the medium containing 50 μg/ml of BPA or BPAF, followed by 9 h in control medium. Oocyte maturity was evaluated by monitoring first polar body extrusion.

Immunostaining of oocytes

Oocyte immunostaining was performed as described previously (Hoshino and Sato, 2008). Briefly, oocytes exposed to the effective concentration of BPA or BPAF (50 μg/ml) were immunostained with α-tubulin and MAD2 antibodies. Oocytes were denuded from the cumulus cells using 0.1% hyaluronidase (Sigma) and pipetting, fixed at room temperature for 60 min in 2% paraformaldehyde (Sigma) in Dulbecco's phosphate-buffered saline (PBS) without magnesium or calcium PBS (Nissui Pharmaceutical, Ueno, Tokyo, Japan) containing 0.1% PVA and 0.2% Triton X-100 (Wako).

The antibodies used were as follows: rabbit monoclonal anti-MAD2 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA; 1:100 dilution); Alexa Fluor 594 donkey anti-rabbit IgG (Molecular Probes, Eugene, Oregano, USA; 1:100 dilution); Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes; 1:100 dilution); and mouse monoclonal anti-α-tubulin (Sigma; 1:200 dilution). Nuclei were labelled using 10 mg/ml Hoechst 33342 (Sigma; 1:100 dilution). Images were acquired using a Zeiss LSM700 confocal microscope.

Statistical analysis

The data were tested statistically using one–way analysis of variance (ANOVA) and Dunnett's test subsequently confirmed homoscedasticity and normality. The analysis software used was SAS 1996 (SAS Institute, USA). Each experiment was repeated three times with at least 30 oocytes. A P-value < 0.05 was considered to be statistically significant.

Results

Effect of BPAF and BPA on oocyte maturation

To analyse the effects of BPA and BPAF on the oocyte maturation, COCs were exposed to 2, 20, 50 or 100 μg/ml BPA and BPAF. Oocytes were cultured for 18 h, the time required to complete oocyte maturation in mice. Compared with controls, cells treated with BPA or BPAF (2 or 20 μg/ml) showed no significant difference in maturity (Fig. 1). However, at concentrations of 50 μg/ml and higher, BPA caused a dose-dependent inhibition of meiotic progress, while BPAF had more potent effects. In the 50 μg/ml BPA-treated group, 45.8% of oocytes reached MII. In comparison, 8.27% of the 50 μg/ml BPAF-treated reached MII. In the groups treated with 100 μg/ml BPA or BPAF, there was also significant decrease in maturation(25.2%, 7.83%).

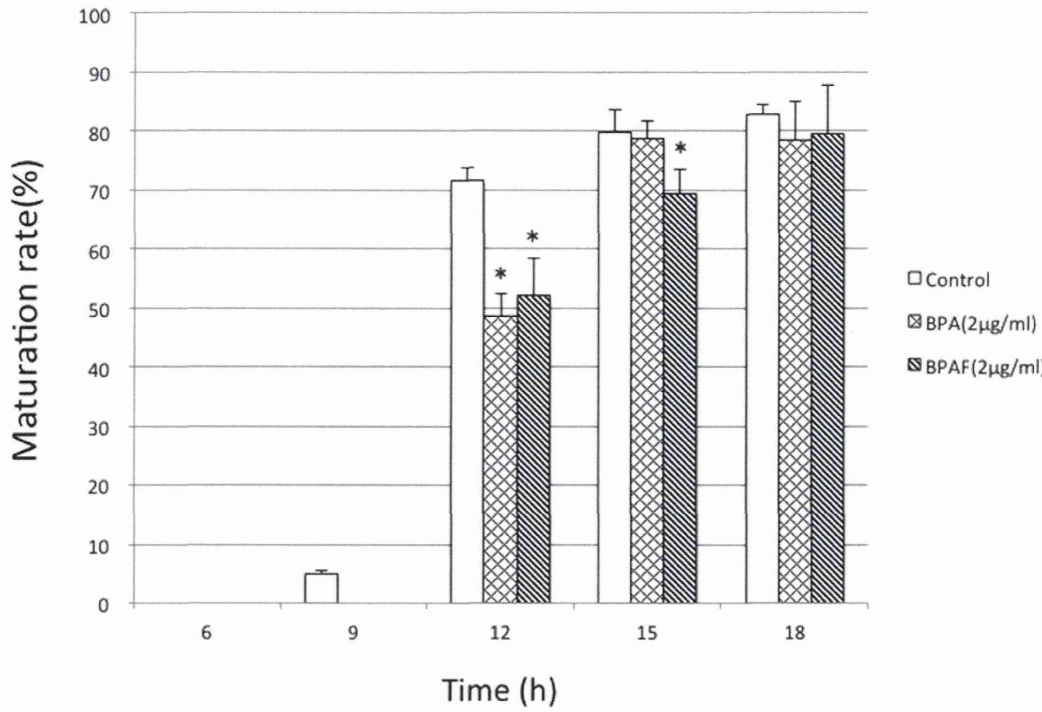

Figure 2 Maturation rate of 2 μg/ml BPA or BPAF-exposed oocytes under various culture conditions. Oocytes were cultured for 6, 9, 12, 15 or 18 h. Values are the means ± standard error of the mean (SEM) of three replicates. Bars with different letters are significantly different (*$P < 0.05$).

Because BPA and BPAF at a dose of 2 μg/ml did not suppress meiotic progression within the 18 h culture period, we analysed the effect of briefer BPA and BPAF exposures on the maturation of oocytes (Fig. 2). COCs were exposed to 2 μg/ml BPA or BPAF and cultured for 6, 9, 12, 15 or 18 h. In the 12 h culture group, BPA- or BPAF-exposed COCs had matured to a lesser extent than controls (48.8 and 51.1%, respectively). However, BPAF-exposed oocytes after 15 h of culture had matured less than BPA-treated oocytes (67.9 and 79.2%, no significant difference).

Effect of high concentrations of BPAF on meiotic progression

To determine whether the meiotic inhibition reported in Fig. 1 was the result of a delay or arrest, COCs were exposed to 50 μg/ml BPA or BPAF for 21 h (Fig. 3). In the BPA-treated group, 46.8 and 81.1% of oocytes reached MII after 18 and 21 h of culture, respectively (Fig. 3). In contrast, 8.5 and 12.3% of BPAF-treated oocytes reached MII after 18 and 21 h of culture.

To determine whether the effect on oocyte maturation was reversible, oocytes were cultured under BPA or BPAF conditions for 12 h and transferred to control medium for 9 h. Under these conditions, 63.3% of BPAF-treated oocytes matured to MII, a percentage that was still significantly lower than the control.

Effect of high concentrations of BPA and BPAF on MAD2 localization

To identify the effect of BPA and BPAF exposure on MAD2 localization in arrested oocytes, treated COCs (50 μg/ml BPA or BPAF, 21 h) were denuded and co-stained using α-tubulin and MAD2 antibodies (Fig. 4). In oocytes exposed to 50 μg/ml BPA, MAD2 was detected not only in the spindle, but also in the cytoplasm. Conversely, in 95% of the oocytes exposed to 50 μg/ml BPAF, MAD2 was detected only in the spindle.

Discussion

BPA is a chemical widely used in the manufacturing of polycarbonate plastics (Ehrlich *et al.*, 2012), epoxy resins, and lacquer lining of food and beverage cans (Bae *et al.*, 2002; Carwile & Michels, 2011). BPA is also used in the fabrication of some dental sealants and composites (Sasaki *et al.*, 2005; Joskow *et al.*, 2006) and has been found in thermal receipt paper (Myers *et al.*, 2009; Biedermann *et al.*, 2010). Notably, from

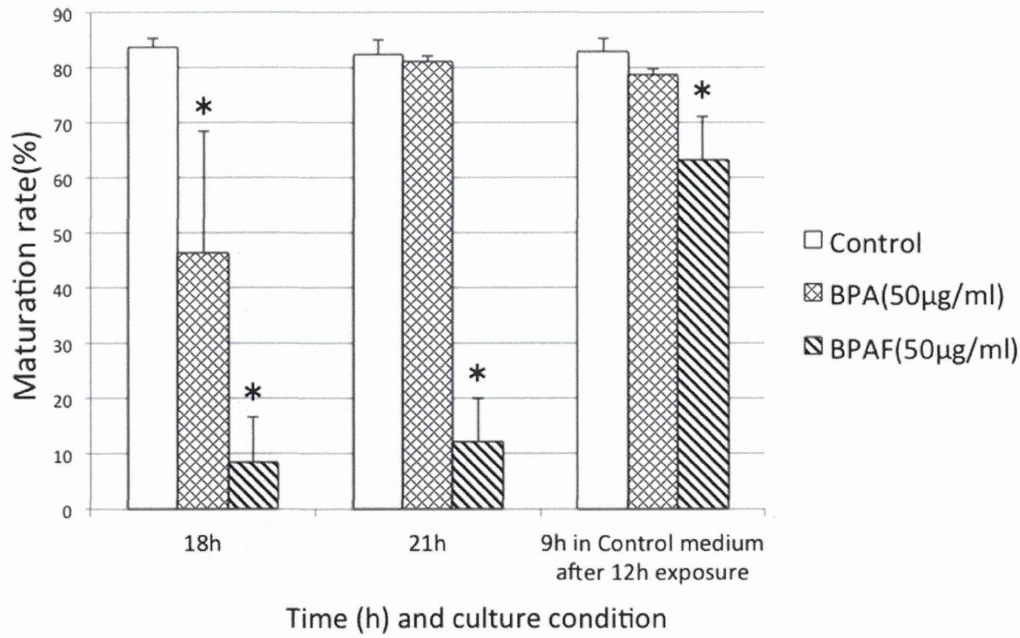

Figure 3 Maturation rate of 50 μg/ml BPA or BPAF-exposed oocytes for 18 h or 21 h, and for 9 h in the control medium after 12 h exposure. Values are the means ± standard error of the mean (SEM) of three replicates. Bars with different letters are significantly different (*$P < 0.05$).

several studies, scientists have reported that these products leach BPA. It has been detected in human serum and poses danger to human health (Hunt *et al.*, 2003; Ikezuki *et al.*, 2002). As alternative to BPA, BPAF is often used. An estimated 10,000–5,000,000 lbs of BPAF are produced annually in the USA (Stout, 2008). Recently, BPAF was nominated for comprehensive toxicological characterization by the US National Institute of Environmental Health Sciences (National Toxicology Program, 2008). However, the information regarding BPAF remains limited. In this study, we analysed the effects of BPA and BPAF on oocyte maturation by altering the concentrations of BPA or BPAF and culture time. The concentration range of BPA and BPAF was selected based on two observations. First, the migration rate of BPA has beem observed to be 2.5 μg/ml. In addition, 45.5 μg/ml of BPA or BPAF fully inhibits microtubule polymerization in cultured V79 cells 140 (Pfeiffer *et al.*, 1997).

Our data indicated that both BPA and BPAF inhibit oocyte maturation (Fig.1). However, BPAF showed sharp reduction in oocytes reaching maturation, whereas BPA led to a dose-dependent reduction. Compared with recent reports, overall oocyte maturation was better in our study. In recent studies, 30 μM (approximately 6.82 μg/ml) and 10 μg/ml doses of BPA led to decreased oocyte maturation (Eichenlaub-Ritter *et al.*, 2008; Lenie *et al.*, 2008). This differed from the culture methods used here. We carried out IVM using a single culture system, a method in which oocytes progressed faster than in mass culture (unpublished data in our laboratory).

BPA reportedly induces meiotic cell cycle delay in mouse oocytes (Can *et al.*, 2005). As show in Fig. 2, we monitored the maturation of oocytes treated with 2 μg/ml BPA or BPAF. As shown in Fig. 1, this concentration had no effect on oocyte maturation. When exposed to 2 μg/ml BPA or BPAF, the majority of oocytes progressed to MII after 15 and 18 h of IVM, respectively. After 12 h of culture, the proportion of BPA- and BPAF-treated oocytes reaching MII (48.5 and 52.2%, respectively) was significantly lower than for the controls (71.6%). Therefore, our data indicate that BPA and BPAF inhibit oocyte maturation; however, at a dose of 2 μg/ml, BPAF is more effective than BPA.

As shown in Fig. 1, oocytes exposed to 50 μg/ml BPA or BPAF for 18 h showed inhibited maturation. Therefore, we examined the extent of maturation in oocytes cultured for 21 h (Fig. 3). The cell cycle was delayed in response 50 μg/ml BPA, whereas oocytes treated with the same concentration of BPAF did not progress to MII, indicating that they had experienced a meiotic arrest.

To analyse the condition of the spindle and SAC in treated oocytes, immunofluorescence staining was performed using antibodies against α-tubulin and MAD2. The exposure to 2 μg/ml BPA and

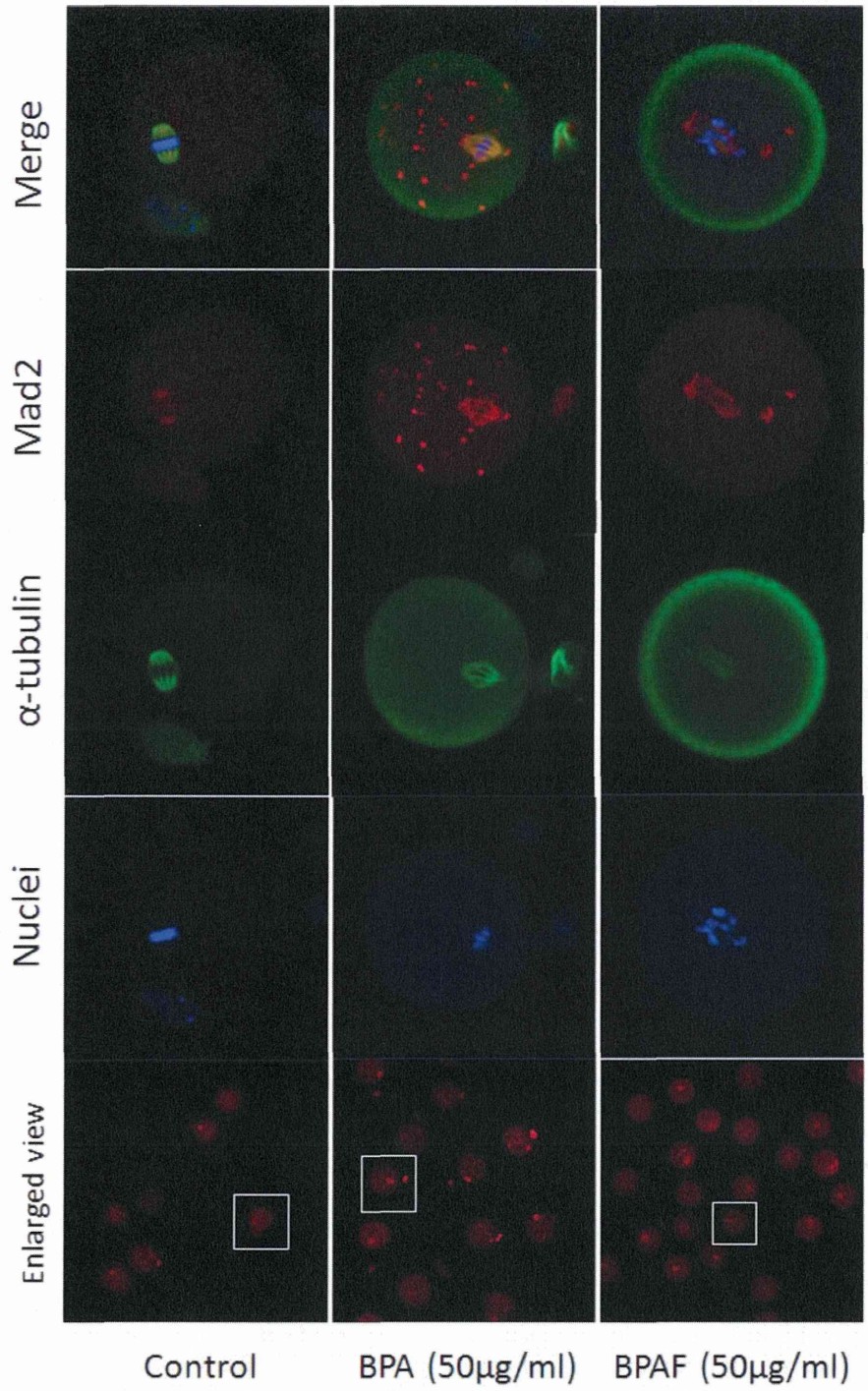

Figure 4 Localization of α-tubulin and Mad2 in oocytes with and without 50 μg/ml of BPA or BPAF treatment.

BPAF caused spindle abnormalities but showed no difference in the MAD2 localization as controls. Consistent with previous reports, 2 μg/ml BPA and BPAF treatments caused spindle abnormalities (Can *et al.*, 2005; Eichenlaub-Ritter *et al.*, 2008; Lenie *et al.*, 2008; Machtinger *et al.*, 2013). Figure 4 shows images of α-tubulin and MAD2 in 50 μg/ml BPA- and BPAF-treated oocytes. In BPAF-treated oocytes (50 μg/ml), which arrest after germinal vesicle breakdown, MAD2 was localized to the spindle. This staining pattern

is reminiscent of oocytes over-expressing MAD2 (Wassmann *et al.*, 2003). The MAD2-dependent SAC is functional during the first meiotic division in mouse oocytes (Niault *et al.*, 2007). Therefore, we speculate that the BPAF-induced meiotic delay and arrest result from SAC activity. Figure 4 showed the localization of MAD2 in BPA-treated oocytes. Multiple small MAD2-positive foci were observed throughout the ooplasm as oocytes progressed to MII. The foci are indicative of SAC dysfunction, which may lead to meiosis delay rather than full arrest. The localization of MAD2 changed with the deformation of the spindle in BPA-treated oocytes.

Here, we report the different effects of BPA and BPAF on oocyte maturation. Our data are consistent with that of previous studies. Because of the CH_3–CF_3 substitution on the bisphenol backbone, differences in receptor selectivity between BPAF and BPA exist. BPA binds strongly to ERRγ, but very weakly to ERα and ERβ (Nagel *et al.*, 1997; Blair *et al.*, 2000; Sheeler *et al.*, 2000; Matsushima *et al.*, 2007). BPAF is a full agonist for ERα and acts as a specific and strong antagonist of the endogenous ERβ agonist 17β-estradiol (Matsushima *et al.*, 2010). These reports suggest that BPAF and BPA have adverse effects on human health.

In conclusion, BPA and BPAF have been shown to act as meiosis-disrupting agents that interfere with microtubule organization and SAC activity. BPA-treated oocytes apparently underwent polar body extrusion normally, but had some abnormalities. Conversely, high concentrations of BPAF arrested oocyte maturation. Thus, we conclude that both BPA and BPAF have toxic effects on reproduction. Also, our laboratory showed that the HD monoculture system is an efficient method to screen substances that affect oocyte culture (Ishikawa *et al.*, 2014; Nishio *et al.*, 2014). The monoculture system can circumvent several problems associated with group culture, a system that is frequently used in artificial reproductive technologies and animal reproduction. The monoculture system requires smaller numbers of oocytes and animals for each experiment thereby making it amenable to high throughput testing. In addition, it is easy to modify and control the microenvironment in monocultures. In this study, we also established that the HD monoculture system, a method in which oocytes have no paracrine effects, is more useful to screen chemicals that effect oocyte maturation.

References

Akahori, Y., Nakai, M., Yamasaki, K., Takatsuki, M., Shimohigashi, Y. & Ohtaki, M. (2008). Relationship between the results of *in vitro* receptor binding assay to human estrogen receptor alpha and *in vivo* uterotrophic assay: comparative study with 65 selected chemicals. *Toxicol. In Vitro* **22**, 225–31.

Bae, B., Jeong, J.H. & Lee, S.L. (2002). The quantification and characterization of endocrine disruptor bisphenol-A leaching from epoxy resin. *Water Sci. Technol.* **46**, 381–7.

Bermudez, D.S., Gray, Jr, L.E. & Wilson, V.S. (2010). Modeling the interaction of binary and ternary mixtures of estradiol with bisphenol A and bisphenol AF in an *in vitro* estrogen-mediated transcriptional activation assay (T47D-KBluc). *Toxicol. Sci.* **116**, 477–87.

Biedermann, S., Tschudin, P. & Grob, K. (2010). Transfer of bisphenol A from thermal printer paper to the skin. *Anal. Bioanal. Chem.* **398**, 571–6.

Blair, R.M., Fang, H., Branham, W.S., Hass, B.S., Dial, S.L., Moland, C.L., Tong, W., Shi, L., Perkins, R. & Sheehan, D.M. (2000). The estrogen receptor relative binding affinities of 188 natural and xenochemicals: structural diversity of ligands. *Toxicol. Sci.* **54**, 138–53.

Bouskine, A., Nebout, M., Brucker-Davis, F., Benahmed, M. & Fenichel, P. (2009). Low doses of bisphenol A promote human seminoma cell proliferation by activating PKA and PKG via a membrane G-protein-coupled estrogen receptor. *Environ. Health Perspect.* **117**, 1053–8.

Can, A., Semiz, O. & Cinar, O. (2005). Bisphenol-A induces cell cycle delay and alters centrosome and spindle microtubular organization in oocytes during meiosis. *Mol. Hum. Reprod.* **11**, 389–96.

Carwile, J.L. & Michels, K.B. (2011). Urinary bisphenol A and obesity: NHANES 2003–2006. *Environ. Res.* **111**, 825–30.

Ehrlich, S., Williams, P.L., Missmer, S.A., Flaws, J.A., Ye, X., Calafat, A.M., Petrozza, J.C., Wright, D. & Hauser, R. (2012). Urinary bisphenol A concentrations and early reproductive health outcomes among women undergoing IVF. *Hum. Reprod.* **27**, 3583–92.

Eichenlaub-Ritter, U., Vogt, E., Cukurcam, S., Sun, F., Pacchierotti, F. & Parry, J. (2008). Exposure of mouse oocytes to bisphenol A causes meiotic arrest but not aneuploidy. *Mutat. Res.* **651**(1–2), 82–92.

Gould, J.C., Leonard, L.S., Maness, S.C., Wagner, B.L., Conner, K., Zacharewski, T., Safe, S., McDonnell, D.P. & Gaido, K.W. (1998). Bisphenol A interacts with the estrogen receptor alpha in a distinct manner from estradiol. *Mol. Cell. Endocrinol.* **142**, 203–14.

Homer, H.A., McDougall, A., Levasseur, M., Murdoch, A.P. & Herbert, M. (2005). Mad2 is required for inhibiting securin and cyclin B degradation following spindle depolymerisation in meiosis I mouse oocytes. *Reproduction* **130**, 829–43.

Hoshino, Y. & Sato, E. (2008). Protein kinase B (PKB/Akt) is required for the completion of meiosis in mouse oocytes. *Dev. Biol.* **314**, 215–23.

Hoyt, M.A. (2001). A new view of the spindle checkpoint. *J. Cell Biol.* **154**, 909–11.

Hunt, P.A., Koehler, K.E., Susiarjo, M., Hodges, C.A., Ilagan, A., Voigt, R.C., Thomas, S., Thomas, B.F. & Hassold, T.J. (2003). Bisphenol a exposure causes meiotic aneuploidy in the female mouse. *Curr. Biol.* **13**, 546–53.

Ikezuki, Y., Tsutsumi, O., Takai, Y., Kamei, Y. & Taketani, Y. (2002). Determination of bisphenol A concentrations in human biological fluids reveals significant early prenatal exposure. *Hum. Reprod.* **17**, 2839–41.

Ishikawa, S., Machida, R., Hiraga, K., Hiradate, Y., Suda, Y. & Tanemura, K. (2014). Hanging drop monoculture for selection of optimal antioxidants during *in vitro* maturation of porcine oocytes. *Reprod. Domest. Anim.* **49**, e26–30.

Joskow, R., Barr, D.B., Barr, J.B., Calafat, A.M., Needham, L.L. & Rubin, C. (2006). Exposure to bisphenol A from bisglycidyl dimethacrylate-based dental sealants. *J. Am. Dent. Assoc.* **137**, 353–62.

Kallio, M., Eriksson, J.E. & Gorbsky, G.J. (2000). Differences in spindle association of the mitotic checkpoint protein Mad2 in mammalian spermatogenesis and oogenesis. *Dev. Biol.* **225**, 112–23.

Kim, H.S., Han, S.Y., Yoo, S.D., Lee, B.M. & Park, K.L. (2001). Potential estrogenic effects of bisphenol-A estimated by *in vitro* and *in vivo* combination assays. *J. Toxicol. Sci.* **26**, 111–8.

Kitamura, S., Suzuki, T., Sanoh, S., Kohta, R., Jinno, N., Sugihara, K., Yoshihara, S., Fujimoto, N., Watanabe, H. & Ohta, S. (2005). Comparative study of the endocrine-disrupting activity of bisphenol A and 19 related compounds. *Toxicol. Sci.* **84**, 249–59.

Kuiper, G.G., Lemmen, J.G., Carlsson, B., Corton, J.C., Safe, S.H., van der Saag, P.T., van der Burg, B. & Gustafsson, J.A. (1998). Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. *Endocrinology* **139**, 4252–63.

Laws, S.C., Carey, S.A., Ferrell, J.M., Bodman, G.J. & Cooper, R.L. (2000). Estrogenic activity of octylphenol, nonylphenol, bisphenol A and methoxychlor in rats. *Toxicol. Sci.* **54**, 154–67.

Lenie, S., Cortvrindt, R., Eichenlaub-Ritter, U. & Smitz, J. (2008). Continuous exposure to bisphenol A during *in vitro* follicular development induces meiotic abnormalities. *Mutat. Res.* **651**(1–2), 71–81.

Liu, X., Matsushima, A., Okada, H., Tokunaga, T., Isozaki, K. & Shimohigashi, Y. (2007). Receptor binding characteristics of the endocrine disruptor bisphenol A for the human nuclear estrogen-related receptor gamma. Chief and corroborative hydrogen bonds of the bisphenol A phenol-hydroxyl group with Arg316 and Glu275 residues. *FEBS J.* **274**, 6340–51.

Ma, W., Zhang, D., Hou, Y., Li, Y.H., Sun, Q.Y., Sun, X.F. & Wang, W.H. (2005). Reduced expression of MAD2, BCL2, and MAP kinase activity in pig oocytes after *in vitro* aging are associated with defects in sister chromatid segregation during meiosis II and embryo fragmentation after activation. *Biol. Reprod.* **72**, 373–83.

Machtinger, R., Combelles, C.M., Missmer, S.A., Correia, K.F., Williams, P., Hauser, R. & Racowsky, C. (2013). Bisphenol-A and human oocyte maturation *in vitro*. *Hum. Reprod.* **28**, 2735–45.

Maney, T., Ginkel, L.M., Hunter, A.W. & Wordeman, L. (2000). The kinetochore of higher eucaryotes: a molecular view. *Int. Rev. Cytol.* **194**, 67–131.

Matsushima, A., Kakuta, Y., Teramoto, T., Koshiba, T., Liu, X., Okada, H., Tokunaga, T., Kawabata, S., Kimura, M. & Shimohigashi, Y. (2007). Structural evidence for endocrine disruptor bisphenol A binding to human nuclear receptor ERR gamma. *J. Biochem.* **142**, 517–24.

Matsushima, A., Liu, X., Okada, H., Shimohigashi, M. & Shimohigashi, Y. (2010). Bisphenol AF is a full agonist for the estrogen receptor ERalpha but a highly specific antagonist for ERbeta. *Environ. Health Perspect.* **118**, 1267–72.

Myers, J.P., vom Saal, F.S., Akingbemi, B.T., Arizono, K., Belcher, S., Colborn, T., Chahoud, I., Crain, D.A., Farabollini, F., Guillette Jr, L.J., Hassold, T., Ho, S.M., Hunt, P.A., Iguchi, T., Jobling, S., Kanno, J., Laufer, H., Marcus, M., McLachlan, J.A., Nadal, A., Oehlmann, J., Olea, N., Palanza, P., Parmigiani, S., Rubin, B.S., Schoenfelder, G., Sonnenschein, C., Soto, A.M., Talsness, C.E., Taylor, J.A., Vandenberg, L.N., Vandenbergh, J.G., Vogel, S., Watson, C.S., Welshons, W.V. & Zoeller, R.T. (2009). Why public health agencies cannot depend on good laboratory practices as a criterion for selecting data: the case of bisphenol A. *Environ. Health Perspect.* **117**, 309–15.

Nagel, S.C., vom Saal, F.S., Thayer, K.A., Dhar, M.G., Boechler, M. & Welshons, W.V. (1997). Relative binding affinity-serum modified access (RBA-SMA) assay predicts the relative *in vivo* bioactivity of the xenoestrogens bisphenol A and octylphenol. *Environ. Health. Perspect.* **105**, 70–6.

National Toxicology Program (NTP) (2008). Chemical information profile for bisphenol AF [CAS No. 1478–61–1], supporting nomination for toxicological evaluation by the National Toxicology Program. from http://ntp.niehs.nih.gov/ntp/htdocs/Chem_Background/ExSumPdf/BisphenolAF_093008_508.pdf

Niault, T., Hached, K., Sotillo, R., Sorger, P.K., Maro, B., Benezra, R. & Wassmann, K. (2007). Changing Mad2 levels affects chromosome segregation and spindle assembly checkpoint control in female mouse meiosis I. *PLoS One* **2**, e1165.

Nishio, M., Hoshino, Y., Tanemura, K. & Sato, E. (2014). Effect of single-oocyte culture system on *in vitro* maturation and developmental competence in mice. *Reprod. Med. Biol.* **13**, 153–9.

Okada, H., Tokunaga, T., Liu, X., Takayanagi, S., Matsushima, A. & Shimohigashi, Y. (2008). Direct evidence revealing structural elements essential for the high binding ability of bisphenol A to human estrogen-related receptor-gamma. *Environ. Health Perspect.* **116**, 32–8.

Pennie, W.D., Aldridge, T.C. & Brooks, A.N. (1998). Differential activation by xenoestrogens of ER alpha and ER beta when linked to different response elements. *J. Endocrinol.* **158**, R11–14.

Pfeiffer, E., Rosenberg, B., Deuschel, S. & Metzler, M. (1997). Interference with microtubules and induction of micronuclei *in vitro* by various bisphenols. *Mutat. Res.* **390**(1–2), 21–31.

Sasaki, N., Okuda, K., Kato, T., Kakishima, H., Okuma, H., Abe, K., Tachino, H., Tuchida, K. & Kubono, K. (2005). Salivary bisphenol-A levels detected by ELISA after restoration with composite resin. *J. Mater. Sci. Mater. Med.* **16**, 297–300.

Shannon, K.B., Canman, J.C. & Salmon, E.D. (2002). Mad2 and BubR1 function in a single checkpoint pathway that responds to a loss of tension. *Mol. Biol. Cell.* **13**, 3706–19.

Sheeler, C.Q., Dudley, M.W. & Khan, S.A. (2000). Environmental estrogens induce transcriptionally active estrogen receptor dimers in yeast: activity potentiated by the coactivator RIP140. *Environ. Health Perspect.* **108**, 97–103.

Stout, M. (2008). National Toxicology Program http://ntp.niehs.nih.gov/

Takayanagi, S., Tokunaga, T., Liu, X., Okada, H., Matsushima, A. & Shimohigashi, Y. (2006). Endocrine disruptor bisphenol A strongly binds to human estrogen-related receptor gamma (ERRgamma) with high constitutive activity. *Toxicol. Lett.* **167**, 95–105.

Wang, W.H. & Sun, Q.Y. (2006). Meiotic spindle, spindle checkpoint and embryonic aneuploidy. *Front. Biosci.* **11**, 620–36.

Wassmann, K., Niault, T. & Maro, B. (2003). Metaphase I arrest upon activation of the Mad2-dependent spindle checkpoint in mouse oocytes. *Curr. Biol.* **13**, 1596–608.

Yang, Y., Yin, J., Yang, Y., Zhou, N., Zhang, J., Shao, B. & Wu, Y. (2012). Determination of bisphenol AF (BPAF) in tissues, serum, urine and feces of orally dosed rats by ultra-high-pressure liquid chromatography-electrospray tandem mass spectrometry. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **901**, 93–7.

Yu, H. (2002). Regulation of APC-Cdc20 by the spindle checkpoint. *Curr. Opin. Cell Biol.* **14**, 706–14.

Zhang, D., Ma, W., Li, Y.H., Hou, Y., Li, S.W., Meng, X.Q., Sun, X.F., Sun, Q.F. & Wang, W.H. (2004). Intra-oocyte localization of MAD2 and its relationship with kinetochores, microtubules, and chromosomes in rat oocytes during meiosis. *Biol. Reprod.* **71**, 740–8.

NOTE *Anatomy*

The effects analysis of two neonicotinoid insecticides on *in vitro* maturation of porcine oocytes using hanging drop monoculture method

Sadamasa ISHIKAWA[1)], Kou HIRAGA[1)], Yuuki HIRADATE[1)] and Kentaro TANEMURA[1)]*

[1)]*Laboratory of Animal Reproduction and Development, Graduate School of Agricultural Science, Tohoku University, Sendai 981–8555, Japan*

(Received 5 January 2015/Accepted 24 January 2015/Published online in J-STAGE 6 February 2015)

ABSTRACT. Acetamiprid (ACE) and imidacroprid (IMI) are known neonicotinoid insecticides with strong affinities for the insect-selective nicotinic acetylcholine receptor. These provide insect control by hyperstimulating insect nerves and are used for agricultural pest management. However, it has also been reported that ACE and IMI affect mammalian reproductive function. We determined the effects of ACE and IMI on the *in vitro* maturation of porcine oocytes. Significant decreases in nuclear maturation rates were observed in the ACE or IMI-exposed groups. Also, in matured oocytes from the ACE or IMI-exposed groups, irregular chromosomes were observed. Our results suggest that ACE and IMI exposure was detrimental to porcine oocytes and the extent of the effects depends on the concentration of exposure.
KEY WORDS: hanging drop, *in vitro* maturation (IVM), neonicotinoid, porcine oocyte

doi: 10.1292/jvms.15-0008; *J. Vet. Med. Sci.* 77(6): 725–728, 2015

Neonicotinoid insecticides show strong affinities for the insect-selective nicotinic acetylcholine receptor (nAChR) and control insects by hyperexcitation of their nerves, and their effective insect control has been used to protect many kinds of crops and animal health [10, 17].

Recently, however, two of the main compounds in neonicotinoid, acetamiprid (ACE) and imidacloprid (IMI), were reported to have comparable effects at the mammalian nAChR, and their safety was called into question [8]. The negative effects of exposure to these compounds are unclear, particularly prenatal exposure.

In the past, there have been some reports of the influence of germ line on nicotine exposure. Specifically, the influence of ACE and IMI in mouse oocytes and human sperm has been reported [2, 20].

Furthermore, it was reported that ACE and IMI affect reproductive function. Kapoor *et al.* (2011) reported changes in ovary morphology following IMI exposure [7].

However, there are few reports about the direct effects on mammalian germ line by ACE and IMI. In later years, Gu *et al.* and Rasgele PG reported the effects of ACE and IMI in mouse sperm, but there are no reports in oocyte that are from a female germ cell [3, 14].

Although there is concern that domestic animals, such as porcine, are exposed to these neonicotinoid insecticides, there are limited data available.

We developed a screening system for high throughput characteristics using the Hanging Drop (HD) monoculture system for analyzing of the effects in one oocyte. The HD method that we used in this study is a culture method that makes use of a small drop in a dish base and inversion of the dish to be used for culture of the embryoid body [9] and ovarian follicle [1]. This method is new and contributed to the screening of effective material for culturing porcine oocytes to *in vitro* maturation (IVM) [6].

In this study, using a screening system, we analyzed the effects of ACE and IMI addition to IVM medium on nuclear maturation in porcine oocytes.

Ovaries were collected from prepubertal gilts at a local slaughterhouse, transported to the laboratory in a container within 2 hr of removal and placed in saline warmed to 37°C. The follicular fluid and porcine oocytes were aspirated from antral follicles (diameter: 3–6 mm) with a 10 ml syringe attached to an 18 gauge needle. Compact cumulus–oocyte complexes (COCs) with uniform ooplasm were selected in phosphate-buffered saline (PBS; Nissui Pharmaceutical, Tokyo, Japan) and supplemented with 0.1% polyvinyl alcohol (PVA; Sigma Chemical, St. Louis, MO, U.S.A.). The culture method referred to the report of Hiraga *et al.* (2013) [5]. After washing three times in 0.1% PBS–PVA, the COCs were cultured in 10 μl (10 μl of medium/1 COC) of NCSU-23 medium [12] supplemented with 50 μM β-mercaptoethanol (Sigma), 0.6 mM cysteine (Sigma), 0.5% insulin (Gibco-BRL, Cergy Pontoise, France; Cat. 18125), 10% (v/v) porcine follicular fluid, 10 IU pregnant mare serum gonadotropin (PMSG; Serotropin; Teikokuzouki, Tokyo, Japan), 10 IU human chorionic gonadotropin (hCG; Puberogen; Sankyo, Tokyo, Japan) and 1 mM dibutyryl cyclic AMP (Sigma) for the first 22 hr of maturation at 38.5°C in 5% CO_2 in air. Then, the COCs were cultured for 22 hr in the medium without hormones or dbcAMP. Each droplet of medium was overlaid with liquid paraffin (NACALAI TESQUE, INC., Kyoto, Japan).

*CORRESPONDENCE TO: TANEMURA, K., Laboratory of Animal Reproduction and Development, Graduate School of Agricultural Science, Tohoku University, Sendai 981–8555, Japan.
e-mail: kentaro@m.tohoku.ac.jp

After 44 hr of incubation, denuded oocytes (DOs) were prepared from some of the incubated COCs by removing cumulus cells through gentle vortexing in PB1 medium [13] that contained 0.1% hyaluronidase (Sigma). DOs were mounted on glass slides and then fixed in acetic acid: ethanol (1:3) for 48 hr. The fixed oocytes were stained with 1% aceto-orcein and observed under a phase-contrast microscope to evaluate nuclear status

We used the HD method for the IVM experiments. In the HD method, 10 μl of the culture medium were placed in each well of a 96-well plate (Thermo Fisher Scientific Inc., Waltham, MA, U.S.A.), and each droplet of medium was overlaid with liquid paraffin. After 1 COC was placed in the medium, the plate was inverted.

COCs were cultured with various concentrations of ACE (Wako Pure Chemical Industries, Ltd., Osaka, Japan) or IMI (Wako) prepared using dimethyl sulfoxide (NACALAI TESQUE) (≤0.1%), at finally determined concentrations (10, 30 and 100 ppm) for 44 hr. After 44 hr of total incubation, all oocytes were fixed. Nuclear status was analyzed, and the percentages of oocyte at MII stage relative to all of the cultured oocytes were calculated.

The range of concentrations of tested chemicals was set on a pesticide residue reference value in Japan, and the minimum of pesticide residue reference value is that ACE is 30 ppm and IMI is 10 ppm. Also, in this study, 100 ppm was set as the maximum.

Each experiment was replicated at least three times. Data are presented as means ± standard error (SE). Statistical analyses were carried out using analysis of variance (ANOVA) and Fisher's protected least significant difference test. A P value <0.01 was considered to indicate significant differences (**P<0.01, *P<0.05).

The results showed that the nuclear maturation rate in the ACE-exposed group was 10 ppm (73.58 ± 4.84%), which was not significantly different from that of the control group (78.46 ± 3.85%). However, in the ACE-exposed groups at 30 ppm and 100 ppm (66.76 ± 4.12%, 53.85 ± 2.32%), a significant decrease in the nuclear maturation rate was observed compared with the control group. Conversely, when the IMI was added at all doses, the nuclear maturation rate was significantly lower than the control (10 ppm: 66.09 ± 5.81%, 30 ppm: 61.17 ± 8.90% and 100 ppm: 56.52 ± 7.53%). Furthermore, when the ACE-exposed group was compared with the IMI-exposed group at 100 ppm, the nuclear maturation of the ACE-exposed group tended to be lower than the IMI-exposed group (P≈0.55) (Fig. 1).

In normal metaphase II oocytes (control group), localized chromosomes of oocyte and polar body were observed as shown in Fig. 2A. But, in metaphase II oocytes which exposed with ACE or IMI groups, the chromosomes were dispersed (irregular chromosomes) (Fig. 2B and 2C). These oocytes were observed in ACE-exposed groups at 30–100 ppm and IMI-exposed groups at 10–100 ppm.

Although it is generally thought that neonicotinoid insecticides have low affinity for mammalian nAChRs, in ACE and IMI, two of the main compounds in neonicotinoid, it has been reported that ACE and IMI affect mammalian nAChR

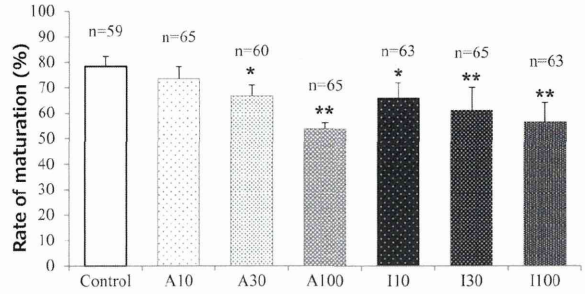

Fig. 1. Effects on nuclear maturation by ACE and IMI (10, 30, 100 ppm) addition to the *in vitro* maturation medium. Data are presented as means ± standard deviations. Different letters indicate significant differences among the treatments. *P<0.05, ** P<0.01.

at the same level as nicotine [8]. In addition, it has also been reported that these have effects on reproduction [3, 14]. Thus, in this study, we evaluated porcine oocytes that were exposed to neonicotinoid insecticides. Finally, we analyzed effects on porcine oocyte IVM from addition of ACE and IMI to IVM medium during nuclear maturation.

Our results show that the nuclear maturation rate of the ACE-exposed group at 10 ppm was not significantly different, although a significant decrease in nuclear maturation rate was observed in the IMI-exposed group at 10 ppm. From the above, we suggest that the threshold of effects of IMI is lower than that of ACE in porcine oocyte IVM. Also, although a significant difference was observed in ACE or IMI-exposed groups at 30 ppm, this difference in IMI-exposed group was bigger than in the ACE-exposed group. Conversely, a significant difference (P<0.01) was observed in the ACE or IMI-exposed groups at 100 ppm, and the nuclear maturation of the ACE-exposed group became lower than the IMI-exposed group. Therefore, we suggest that nuclear maturation is inhibited in ACE at more than 30 ppm, and in IMI at more than 10 ppm. It is likely that ACE has more detrimental effects than IMI above 100 ppm. Additionally, an irregular chromosome was observed in metaphase II oocytes for ACE and IMI-exposed groups. The IMI-exposed group showed these effects at a lower concentration (10 ppm) than the ACE exposed group (30 ppm). We suggest that IMI is more detrimental than ACE, at low concentrations in porcine oocyte IVM.

The mechanism of the effects of ACE and IMI on porcine oocyte maturation is not clear. However, in rat ovaries from animals exposed to IMI by oral administration, a significant increase of lipid peroxide (LPO) is caused by decreases of the antioxidant glutathione and antioxidative enzyme activity including superoxide dismutase, catalase and glutathione peroxidase [7]. The huge amount of lipid in a porcine oocyte, compared with oocytes from other mammalian species, is mostly made up of triglycerides (TGs) [11, 15, 16]. TG plays an important role in energy metabolism by providing adenosine triphosphate (ATP) for protein synthesis, meiosis and cytoplasmic maturation [15]. In addition, we recognize that TG is oxidized to LPO [4]. LPO generally damages

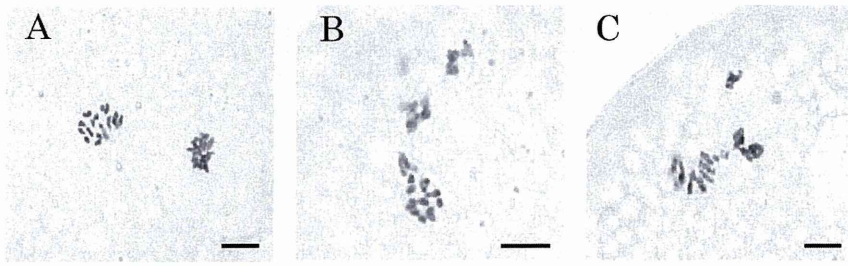

Fig. 2. Chromosomes in metaphase II oocytes. (A) control, (B) acetamiprid exposed groups, (C) imidacloprid exposed groups (Orcein staining). Bar=5 μm

DNA and is known as a cause of cancer [19]. Also, Yazaki *et al.* reported that the nuclear maturation rate becomes lower as the ratio of LPO in porcine oocyte increases [18]. We reported that a significant increase of nuclear maturation was caused by addition of antioxidants to IVM medium in porcine oocyte IVM [6], suggesting the damage of oxidative stress on porcine oocyte IVM is serious.

Therefore, the decrease of nuclear maturation was likely caused by the influence of oxidative stress, from TG becoming oxidized to LPO in oocytes by direct exposure to IMI. However, in ACE and IMI, it is thought that a more detailed influence analysis using porcine oocyte is necessary.

The HD monoculture system used for this study had high-throughput efficiency, and we succeed in rapid screening of effective material in porcine oocytes on IVM [6]. We screened ACE and IMI as these are believed to have adverse effects that can be detected using the HD monoculture system. As a result, we could detect reproductive toxicity that inhibited nuclear maturation in porcine oocyte. Therefore, the screening system was useful for assessing the effects of exposure to chemical substances in porcine oocyte IVM. We suggest this screening system could contribute to livestock production and improve the productivity of domestic animal agriculture by functioning as an index of safety assessment for agricultural chemicals.

ACKNOWLEDGMENTS. We thank the staff of Meat Inspection Office, Sendai, Japan, for supplying the porcine ovaries.

REFERENCES

1. Choi, J. K., Agarwal, P. and He, X. 2013. *In vitro* culture of early secondary preantral follicles in hanging drop of ovarian cell-conditioned medium to obtain MII oocytes from outbred deer mice. *Tissue Eng. Part A* **19**: 2626–2637. [Medline] [CrossRef]
2. Gandini, L., Lombardo, F., Lenzi, A., Culasso, F., Pacifici, R., Zuccaro, P. and Dondero, F. 1997. The *in-vitro* effects of nicotine and cotinine on sperm motility. *Hum. Reprod.* **12**: 727–733. [Medline] [CrossRef]
3. Gu, Y. H., Li, Y., Huang, X. F., Zheng, J. F., Yang, J., Diao, H., Yuan, Y., Xu, Y., Liu, M., Shi, H. J. and Xu, W. P. 2013. Reproductive effects of two neonicotinoid insecticides on mouse sperm function and early embryonic development in vitro. *PLoS ONE* **8**: e70112. [Medline] [CrossRef]
4. Gutteridge, J. M. 1994. Biological origin of free radicals, and mechanisms of antioxidant protection. *Chem. Biol. Interact.* **91**: 133–140. [Medline] [CrossRef]
5. Hiraga, K., Hoshino, Y., Tanemura, K. and Sato, E. 2013. Selection of *in vitro*-matured porcine oocytes based on localization patterns of lipid droplets to evaluate developmental competence. *J. Reprod. Dev.* **59**: 405–408. [Medline] [CrossRef]
6. Ishikawa, S., Machida, R., Hiraga, K., Hiradate, Y., Suda, Y. and Tanemura, K. 2014. Hanging drop monoculture for selection of optimal antioxidants during in vitro maturation of porcine oocytes. *Reprod. Domest. Anim.* **49**: e26–e30. [Medline] [CrossRef]
7. Kapoor, U., Srivastava, M. K. and Srivastava, L. P. 2011. Toxicological impact of technical imidacloprid on ovarian morphology, hormones and antioxidant enzymes in female rats. *Food Chem. Toxicol.* **49**: 3086–3089. [Medline] [CrossRef]
8. Kimura-Kuroda, J., Komuta, Y., Kuroda, Y., Hayashi, M. and Kawano, H. 2012. Nicotine-like effects of the neonicotinoid insecticides acetamiprid and imidacloprid on cerebellar neurons from neonatal rats. *PLoS ONE* **7**: e32432. [Medline] [CrossRef]
9. Lee, W. G., Ortmann, D., Hancock, M. J., Bae, H. and Khademhosseini, A. 2010. A hollow sphere soft lithography approach for long-term hanging drop methods. *Tissue Eng. Part C Methods* **16**: 249–259. [Medline] [CrossRef]
10. Matsuda, K., Buckingham, S. D., Kleier, D., Rauh, J. J., Grauso, M. and Sattelle, D. B. 2001. Neonicotinoids: insecticides acting on insect nicotinic acetylcholine receptors. *Trends Pharmacol. Sci.* **22**: 573–580. [Medline] [CrossRef]
11. McEvoy, T. G., Coull, G. D., Broadbent, P. J., Hutchinson, J. S. and Speake, B. K. 2000. Fatty acid composition of lipids in immature cattle, pig and sheep oocytes with intact zona pellucida. *J. Reprod. Fertil.* **118**: 163–170. [Medline] [CrossRef]
12. Petters, R. M. and Wells, K. D. 1993. Culture of pig embryos. *J. Reprod. Fertil. Suppl.* **48**: 61–73. [Medline]
13. Quinn, P., Barros, C. and Whittingham, D. G. 1982. Preservation of hamster oocytes to assay the fertilizing capacity of human spermatozoa. *J. Reprod. Fertil.* **66**: 161–168. [Medline] [CrossRef]
14. Rasgele, P. G. 2014. Abnormal sperm morphology in mouse germ cells after short-term exposures to acetamiprid, propineb, and their mixture. *Arh. Hig. Rada Toksikol.* **65**: 47–56. [Medline] [CrossRef]
15. Sturmey, R. G. and Leese, H. J. 2003. Energy metabolism in pig oocytes and early embryos. *Reproduction* **126**: 197–204. [Medline] [CrossRef]
16. Sturmey, R. G., Reis, A., Leese, H. J. and McEvoy, T. G. 2009. Role of fatty acids in energy provision during oocyte maturation and early embryo development. *Reprod. Domest. Anim.* **44** Suppl 3: 50–58. [Medline] [CrossRef]
17. Tomizawa, M. and Casida, J. E. 2005. Neonicotinoid insecticide