Songklanakarin J. Sci. Technol.
41 (5), 1029-1036, Sep. - Oct. 2019

http://www.sjst.psu.ac.th

Original Article

Porcine cumulus oocyte complexes (pCOCs) as biological model for determination on in *vitro* cytotoxic of cadmium and copper assessment

Waramanee Pongsawat and Mayuva Youngsabanant*

Department of Biology, Faculty of Science,
Silpakorn University, Sanamchandra Palace Campus, Mueang, Nakhon Pathom, 73000 Thailand

Received: 24 September 2017; Revised: 27 May 2018; Accepted: 31 May 2018

Abstract

We demonstrated the potential of primary porcine cumulus oocyte complexes (pCOCs) used as a biological model for determination of *in vitro* toxicity of cadmium and copper. After treatment at a concentration of 10^{-4} M of cadmium and copper for 44 h, the surrounding cumulus cells (CCs) stopped the expansion process and the pCOCs did not undergo maturation in either treatment. Compared to the control, degradation of the pCOCs was observed as well as a number of vacuoles associated with metal accumulation. Cumulus cells and zona-pellucida revealed a rough surface with numerous knobs. The cumulus cells showed a loss of cell margin and signs of cytoplasmic organelle damage. The percent adsorption rate of cadmium and copper from inductively coupled plasma were 5.65 and 6.85, respectively, and there was no significant difference between treatment groups ($P>0.05$). This finding is valuable for future cell technology research in medical and *in vitro* cytotoxicity studies.

Keywords: cadmium, copper; cytotoxicity, porcine cumulus oocyte complexes (pCOCs), electron microscopy

1. Introduction

Cadmium is a toxic heavy metal that contaminates the environment, e.g., water, air, and plants (Nad *et al.*, 2007; Satarug & Moore, 2004). It can be taken up into plants or enter animal tissues through food chains such as contaminated water or food. In general, cadmium absorption after dietary exposure in humans is relatively low. The absorption rate of cadmium in the gastro-intestinal tract depends on the species, type of cadmium compound, dose size and frequency, age, and interaction with various dietary components such as calcium, iron, magnesium, zinc, copper, and protein. Cadmium uptake is unaffected by the presence of calcium ions. However, the toxicity of cadmium is enhanced by ingestion of a low calcium diet even when consumption of diet is equalized. The mechanism of cadmium absorption involves calcium-binding protein (CaBP) and uptakes into the mucosal cells. The fate of intramucosal cadmium is either transport into the circulation or sequestration to an induced ligand such as metallothionein. Feeding a low calcium diet appears to favor the transport process thereby facilitating the absorption of this toxic metal (Verbost, Flik, Lock, & Wandelaar Bonga, 1988; Washko & Cousins, 1977). Accumulation of cadmium in the body at high levels can severely damage lung, liver, kidney, bone, circulatory, and immune systems. At the cellular level, cadmium can affect cell proliferation and cell cycle progression (Templeton & Liu, 2010), DNA repair (Bertin & Averbeck, 2006), and cell-cell adhesion (Prozialeck *et al.*, 2008). Cadmium may induce cell death via the apoptosis pathway. Exposure to cadmium is known to cause endoplasmmic reticulum (ER) stress leading into loss of organelle function and activity (Nordberg, Herber, & Alessio, 1992; Wang *et al.*, 20 09; World Health Organization [WHO], 1992). Several publications have reported the contamination of cadmium in aquatic sources near industries through industrial waste water, in which this heavy metal consequently poisoned people through consumption of contaminated seafood, e.g., fishes or shells (Ahmed *et al.*, 2016; Leoni *et al.*, 2002; Thompson & Bannigan, 2008; WHO, 1992). In addition to consumption,

*Corresponding author
Email address: maijackee@hotmail.com

cadmium uptake from smoking or inhalation of cadmium containing fumes has been studied. It was found that cadmium may exert effects on the reproductive system (Aprioku, Maxwell, & Ijomah, 2014; Satarug & Moore, 2004; Thompson & Bannigan, 2008) and induce chromosomal changes which consequently reduce the fertilization rate in organisms (Watanabe & Endo, 1997).

Copper is an element that occurs in natural systems and is an essential trace element for normal growth. It is also a component of several enzymes or acts as a cofactor in human metabolism, catalysts, and enzyme activators (Dobrzański, Kołacz, & Bodak, 1996). Copper deficiency, which is indicated by symptoms of leukopenia and osteoporosis, causes homeostatic imbalance. It is an essential trace mineral for some organisms but exposure to high doses may have certain effects on organisms. Toxicity of copper depends on the species, period of time, and concentration of copper uptake into the body where it accumulates in the liver and reproductive organs (Stawarz, Zakrzewski, Marenčík, & Hraška, 2003). In humans, copper can enter the body by drinking water or eating food that was collected from environments contaminated with copper metal or copper compounds in pesticides, cans, and electronic equipment (Georgopoulos, Roy, Yonone-Lioy, Opiekun, & Lioy, 2001). An example is copper sulfate, which is often used for control of algal blooms. It was reported that copper is toxic to humans at a minimum copper intake of 11 mg/kg (Clayton & Clayton, 1981). Therefore, people can be at risk of copper toxicity in the environment if exposed at high doses, (Hodson, Borgmann, & Shear, 1979; Wright & Welbourn, 2002). This work focused on the *in vitro* effects of cadmium and copper on mammalian cells. Since there are many pig farms and slaughterhouses located in Nakorn Pathom Province, Thailand, it is an important economic animal in this area. For this reason, porcine cumulus oocyte complexes (pCOCs) can be easily collected from ovaries because they are not used as human food. We have previously proposed that pCOCs can be used as an experimental model (Sanmanee & Areekijseree, 2009, 2010). In this study, the morphological characteristics of pCOCs after treatment with cadmium and copper were investigated using transmission and scanning electron microscopy. The results confirmed that pCOCs can be efficiently utilized in lieu of experimental animals. The fact that both pigs and humans are mammals, it is possible that all consequences, due to cadmium and copper that occur in pCOCs would reflect similar effects of metal toxicity on humans. Accordingly, this study demonstrated the application of pCOCs maintained in a primary culture condition as a model animal to assess the *in vivo* and *in vitro* effects of cadmium and copper toxicity on primary pCOCs.

2. Materials and Methods

2.1 Porcine ovary collection and preparation

One hundred and twenty ovaries of reproductive Large White pigs, at the ages of 210-250 days old were collected from local slaughterhouses, kept in 0.9% normal saline supplemented with 100 IU/mL penicillin, 100 µg/mL streptomycin, and 250 µg/mL Fungizone® and maintained at 30-35 °C during transportation to the laboratory. The samples were used for experiment within 1 h after collection. At the laboratory, the ovaries were washed 3 times with 0.9% normal saline supplemented with gentamicin (50 µg/mL). pCOCs were aspirated by puncturing the ovarian follicles (2-8 mm in diameter) with an 18 gauge needle attached to a syringe filled with washing medium. For preparation of the pCOCs, follicular content was observed under a stereomicroscope and the pCOCs were collected using a pipette of narrow pore size (200-220 µm). After aspiration, the pCOCs were washed 3 times with a washing medium. The pCOCs was then classified into four types described by Areekijseree and Vejaratpimol (2006).

The pCOCs were recovered and measured under a stereomicroscope in order to categorize the groups based on the number of cumulus cell (CC) layers surrounding the oocyte. The pCOCs were classified into four types. Type I pCOCs were intact cumulus cell layers surrounding the oocyte, i.e. the oocytes contained compact CCs of more than 5 layers. Type II pCOCs were multi-cumulus cell layers sur-rounding oocyte, i.e. the oocytes were surrounded with 2-3 incomplete layers of CCs. Type III pCOCs were partial CC layers that surrounded the oocyte, i.e. the oocytes were par-tially covered with some CC layers. Type IV pCOCs were completely denuded oocytes, i.e. the oocytes were completely denuded of CCs. Type I and Type II pCOCs were washed an additional 5 times with washing medium. Ninety of Type I and Type II pCOCs were used for the morphology study under inverted microcopy and electron microscopy and 270 of Type I and Type II pCOCs were used for cytotoxicity assessments.

2.2 Porcine cumulus oocyte complexes preparation for morphology study by scanning electron microscopy (SEM)

The pCOCs were washed 3 times with 0.1M phosphate buffer (pH 7.2), and fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.2). They were then washed 3 times (10 min each) with 0.1 M phosphate-buffer at pH 7.2 and dehydrated in a graded series of ethanol (30, 50, 70, 80, 90, and 100%). Subsequently, the samples were dried in a critical point dryer machine and mounted on stubs with conductive carbon tape, coated with gold particles at 20 nm thick in anion sputtering. All samples were observed and examined under SEM (CamScan Analytical, Maxim 2000S) operating at 15 kV.

2.3 Culture medium for cytotoxicity assessment on pCOCs

The standard culture medium consisted of M199 medium with Earle's salts (Sigma-Aldrich, Saint Louis, MO, USA) supplemented with 10% heat-treated fetal bovine serum (HTFCS), 2.2 mg/mL $NaHCO_3$ (Sigma Company, St. Louis, MO), 0.25 mM pyruvate, 15 µg/mL porcine follicle stimulating hormone, 1 µg/mL leutinizing hormone, 1 µg/mL estradiol with ethanol, and 50 µg/mL gentamycin sulfate. The culture medium was equilibrated at a high humidified atmosphere with 5% CO_2 in 95% air atmosphere at 37 °C for 24 h before use.

2.4 Cytotoxicity assessment on pCOCs

2.4.1 Cadmium preparation (Sanmanee & Areekijseree, 2009)

Cadmium chloride (CdCl2·2.5H2O) obtained from Sigma; CAS no. 7790-78-5 was freshly prepared from stock. Cd^{2+} was prepared at a concentration of 10^{-1} M and diluted with deionized water (Nano Pure) to 10^{-3} M. One milliliter of Cd^{2+} (10^{-3} M) was withdrawn and adjusted to 10 mL with deionized water to make 10^{-4} M Cd^{2+}.

2.4.2 Copper preparation (Sanmanee & Areekijseree, 2010)

Copper chloride (CuCl2·2.H2O) was prepared as a stock solution at a concentration of 10^{-1} M. Then, it was diluted with deionized water (Nano Pure) to 10^{-3} M. One milliliter of 10^{-3} M Cu^{2+} was withdrawn to make a concentration of 10^{-4} M to the final volume of 10 mL.

2.4.3 Cytotoxicity assessment of cadmium and copper on pCOCs (Leoni *et al.*, 2002)

To assay the cytotoxicity, pCOCs were separated into 3 groups: a control group, 10^{-4} M $CdCl_2$ treatment, and 10^{-4} $CuCl_2$ treatment. Each group was composed of 30 pCOCs (Types I and II) and cultured in a small petri dish (35x15 mm) that contained about 2 mL of M 199, supplemented with 10% HTFCS. The cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 for 44 h. After incubation, pCOCs were washed 3 times with milli-Q water to remove the non-absorbed metal. Each group was then divided into 2 subgroups. Subgroup I pCOCs were morphologically studied by transmission electron microscopy (TEM). In the subgroup II pCOCs, metal accumulation was determined by the inductively coupled plasma. Each experiment was done in triplicate. The statistical comparisons of absorbed cadmium and copper were done using the SPSS program (version 16).

2.4.4 Porcine cumulus oocyte complexes preparation for morphology study by transmission electron microscopy (TEM)

The control and treated pCOCs groups were washed with 0.1 M phosphate buffer (pH 7.2) 3 times before being fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.2) for 24 h. After fixation, the oocytes were washed with 0.1 M phosphate buffer and centrifuged at 900*g*, at 4 °C, 10 min, 3 times. After post-fixing in 1% osmium tetroxide in the same buffer for 2 h, all samples were washed 3 times with 0.1 M phosphate buffer (pH 7.2) following dehydration through a graded series of ethanol (30, 50, 70, 80, 90, and 100%) and embedded in Spurr's resin. The specimens were cut into 1-2 µm thickness and stained with toluidine blue before examining under a light microscope to identify suitable areas. The selected areas of tissue were ultra-thin sectioned to 60-90 nm thickness, stained with 5% uranyl acetate, and 0.4% lead citrate and finally examined under TEM (JEOL 1230) operating at 80 kV.

3. Results

3.1 Morphological study of the pCOCs

The pCOCs used in this study were collected by aspirating the yellow follicular fluid from the follicles of porcine ovaries. Examination of the follicular fluids under inverted and scanning electron microscopes showed zona pellucida, numerous cumulus cells, and oocytes contained in the fluid. A total of 1,923 oocytes were isolated from 120 ovaries which was an average of 16.03 oocytes per ovary. The pCOCs were then classified into i) intact cumulus cell layers, ii) multi-cumulus cell layers, iii) partial cumulus cell layers, and iv) completely denuded oocyte with percentages of 32.71%, 22.83%, 18.56%, and 25.90%, respectively (Figure 1).

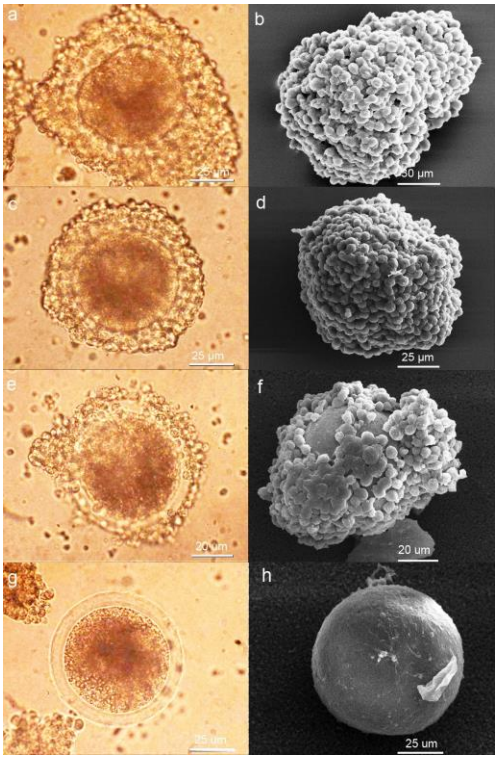

Figure 1. Micrographs and SEM micrographs of an oocyte with intact cumulus cell layers (a-b), an oocyte with multi-cumulus cell layers (c-d), an oocyte with partial cumulus cell layers (e-f), a completely denuded oocyte (g-h) showing the zona pellucida (Z) and cumulus cells (C).

Intact- and multi-cumulus cell layer oocytes were cultured in M199 supplemented with 10% heat inactivated fetal calf serum and hormones for 44 h, which is the maturation period of *in vitro* porcine oocytes. After the incubation time, the process of COC expansion, as well as attachment of the complexes to the bottom of the petri dish and well defined morphological changes of CCs from round to teardrop-like shape, was clearly seen in all pCOCs (Figure 2a-b). In contrast, the pCOCs treated with cadmium concentrations of 10^{-4} M for 44 h showed less expansion (Figure 2c-d) whereas no expansion was observed in the case of pCOCs treated with copper at a concentration of 10^{-4} M for 44 h (Figure 2e-f) and the pCOCs did not undergo oocyte maturation.

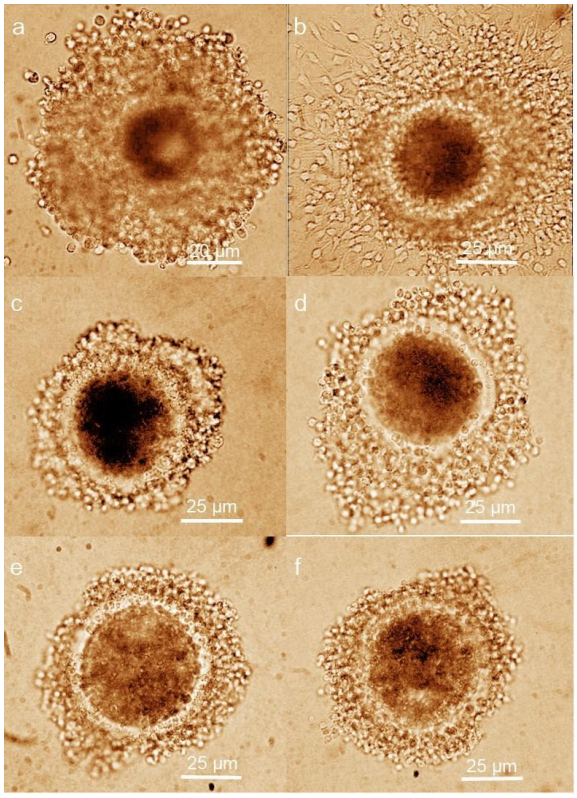

Figure 2. Micrographs of the pCOCs in the control group (culture in medium supplemented with 10% HTFCS and hormones for 44 h) showed round-shaped oocytes with CC expansion (a-b). Porcine COCs treated with cadmium at concentrations of 10^{-4} M for 44 h showed less expansion (c-d), and treated with copper at a concentration of 10^{-4} M (e-f) for 44 h showed no expansion and did not undergo maturation of the pCOCs.

Examination by TEM of the pCOCs cultured in M199 for 44 h showed that the CCs expanded to attach to the surface where the cells were changing from round to teardrop-like shape. In Figure 3, the cells contained an oval nucleus and many organelles in the cytoplasm including mitochondria and ribosome were in good condition. Also, attachments of the margin of cell membrane and the endoplasmic reticulum to the outer nuclear membrane were clearly visible. In addition, connections between the CCs surrounding the oocyte via the gap junction were observed. The connection between the CCs surrounding the oocyte showed secretory vesicles and mitochondria in the cytoplasm of the CCs (Figure 3c-d).

Examination of the pCOCs under SEM revealed various sizes of CCs that appeared in spherical shapes but the cell surfaces were rough. Observation of the zona pellucida found numerous semidome structures on the surface (Figure 4). To investigate the effects of metal toxicity, the pCOCs were cultured in M199 that contained cadmium at a concentration of 10^{-4} M and cultured in M199 that contained copper at a concentration of 10^{-4} M for 44 h prior to observation under SEM. Compared with the control group, the expansion process of the cadmium treated pCOCs was to a lesser degree and the CCs changed morphologically into teardrop-like shapes.

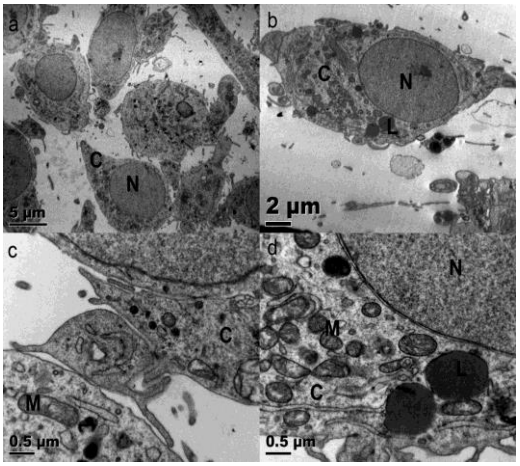

Figure 3. TEM micrographs of ultrastructure of the pCOCs in the control group cultured in medium supplemented with 10% HTFCS and hormones for 44 h showed expansion of CCs in teardrop-like shape (a-b), expanding CCs with nucleus (N), cytoplasm (C), lipid droplet (L), mitochondria (M), and endoplasmic reticulum (Er). The connection between the CCs surrounding the oocyte (c-d) showed secretory vesicle (S), mitochondria (M) in the cytoplasm (C) of the CCs.

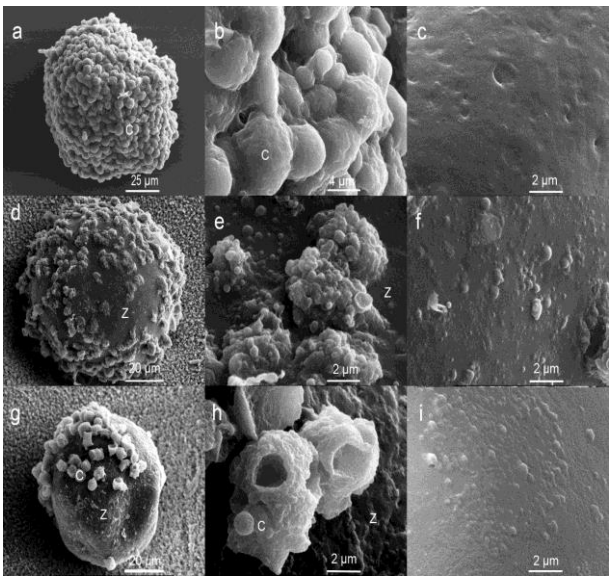

Figure 4. SEM micrographs of ultrastructure of the pCOCs showed normal character in control group cultured in medium supplemented with 10% HTFCS and hormones for 44 h (a-c). Cadmium treatment group at concentrations of 10^{-4} M (d-f) and copper treatment group at concentrations of 10^{-4} M (g-i) showed abnormal cell surfaces. The zona pellucida (Z) showed numerous knobs that increased at the surface area and the surfaces of the CCs (C) were rough and porous.

Treatment by copper under the culture conditions for 44 h caused similar effects to the pCOCs in which expansion of the CCs was to a lesser degree than the control. Thick sections of the pCOCs illustrated distribution of lipid droplets inside the cells. That was associated with accumulation of

SEM study on pCOCs showed that compared with control and treatment groups the CCs contained rougher and more convoluted surface with several pores. Moreover, the cells were irregular and of various sizes (Figure 4e-f). The ultrastructural features of the pCOCs treated with copper were similar to the features seen in the cadmium treatment group. The oocyte clearly underwent a degradation process while the surrounding CCs appeared to detach from the oocyte (Figure 4g-h). Investigations of the pCOCs treated by cadmium and copper using TEM indicated that the oocytes of the two treated groups showed normal morphology character compared with the control group (Figures 5b, 5d, and 5f). Meanwhile the CCs of the two treated groups had damaged nuclei and cytoplasm. Although the inside of the cells had remaining particles associated with each organelle, it appeared to be deforming (Figures 5c and 5e).

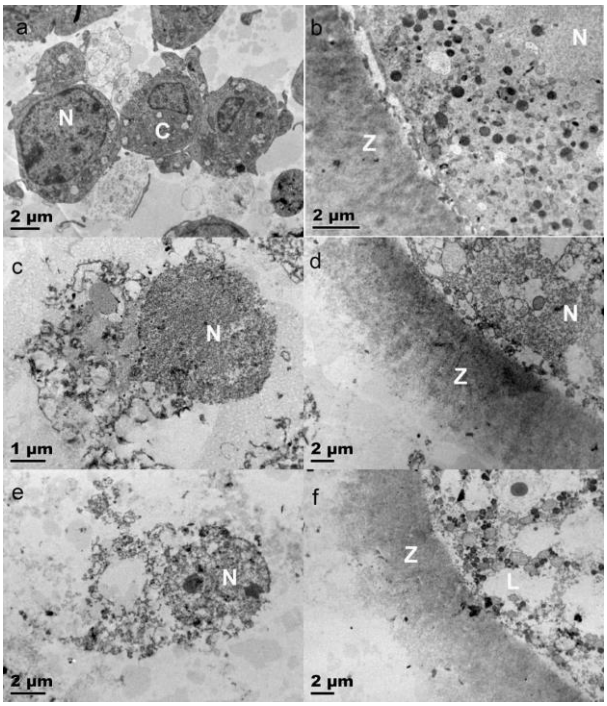

Figure 5. TEM micrographs of the pCOCs showed normal character in control group cultured in medium supplemented with 10% HTFCS and hormones for 44 h (a-b). pCOCs treated with cadmium at concentrations of 10^{-4} M. (c-d) and pCOCs treated with copper at concentrations of 10^{-4} M. (e-f) for 44 h showed damaged nuclei (N) and cytoplasm of the CCs (C), zona pellucida (Z), and numerous lipid droplets (L).

3.2 Cytotoxicity assay of heavy metal on primary cells cultured of pCOCs

After the 44-h culture, the cadmium and copper contents in the culture medium were then determined by inductively coupled plasma analysis. The results showed that the initial and final concentrations were not significantly different between the treatment groups ($P>0.05$). The percentages of adsorption of cadmium and copper were 5.65 and 6.85, respectively.

4. Discussion

In this study, pCOCs were characterized into four types on the basis of accumulation and arrangement of the CCs around the oocytes. The consistent mixing of these four types of pCOCs was found in follicular fluid samples collected from several follicle sizes. However, these four types of pCOCs could be used selectively for different experimental purposes. As for culturing oocyte cells to reach maturation, Mori, Amano, and Shimizu (2000) found that the intact cumulus cell layer type and multi-cumulus cell layer type had higher potential to become mature oocytes. Two types of pCOCs were successfully cultured in culture medium supplemented with follicular stimulating hormone and leutinizing hormone using cell samples from rat (Magnusson, 1980), sheep (Staigmiller & Moor, 1984), bovine, swamp buffalo (Kitiyanant, Tocharus, Areekijseree, & Pavasuthipaisit, 1995), and pig (Areekijseree & Chuen-Im 2012; Areekijseree & Veerapraditsin, 2008).

The toxin absorption and transportation process of toxic heavy metals across cell membranes is associated with the phospholipid bilayers of the cell membrane. The phospholipids are stacked with the non-polar hydrocarbon chains pointed inward while the polar ends act as the external surface. The lipids found on the membrane consist of a hydrophilic (water soluble) part and hydrophobic (water insoluble) part which is a non-polar long hydrocarbon chain of two fatty acids. The fatty acids are present as esters bonded to glycerol. The third-OH group on the glycerol is an ester bonded to phosphate hence the term phospholipid. The proteins are embedded in the membrane and some of which form channels where other molecules can pass through (Williams, James, & Robert, 2000). For this reason, copper and cadmium, which can dissolve in water, move from the high to low solute concentrations (simple diffusion). Compared between the two heavy metals, cadmium has a smaller ionic radius than calcium. It is believed that this ion may function as a homologue of calcium ion at certain types of calcium channels in mammalian cells since it was shown to be an open channel blocker for L-type Ca^{2+}channels in excitable cells (Kuo & Hess, 1993). According to the findings of Blazka and Shaikh (1991), they believed that cadmium may cross the hepatocyte membranes through L-type Ca^{2+}channels.

One type of passive transport is diffusion (or facilitated transport), a form of passive transport facilitated by transport proteins (Fieten, Leegwater, Watson, & Rothuizen, 2012). Cadmium or copper ions diffuse by attaching to a carrier protein. Both of these metal ions can easily bind to both sulfhydryl groups of proteins and the hydroxyl part of phospholipids (Devi & Prasad, 1999). Since cadmium and copper ions can replace calcium ions at its essential sites on the membranes (Breckle & Kahle, 1991), the competitive binding of metal ions to the membrane may cause active ion uptake and ionic homeostasis damage (Salt, Smith, & Raskin, 1998). Toraason and Foulkes (1984) studied ion uptake in rat duodenum and they suggested that calcium and cadmium ions compete for intestinal transport. At the concentrations of 10 µM and 100 µM cadmium, both can block active transport and water transport causing cellular metabolic stop. Eventually, the cells become poisoned by their own toxic waste and die.

In our study, observation of the oocyte by SEM revealed that at the cell surface of zona pellucida numerous knobs were shown to increase on the surface area and the surface of the CCs were rough and porous. This possibly resulted from metabolic wastes that could not be eliminated from the cells due to a malfunction of the transportation process of the cell membrane and became sources of toxicity. However, the binding of cadmium or copper to a membrane is a reversible reaction and unstable over time (Portillo, 2000; Schaller & Sussman, 1988). For this reason, the initial and final concentrations of cadmium and copper ions in the medium after culturing the pCOCs for 44 h showed no significant difference between the treatment groups ($P>0.05$).

Considered the cellular mechanisms of cadmium excretion systems, the excretion process is an ATP-driven primary active transport process. Cadmium is a highly toxic metal that decreases the cellular ATP levels and disturbs the homeostasis of ions leading to damage and changes in cell structure and metabolism which are the reasons the cells cannot get rid of cadmium.

The results showed CC damage and loss of the nuclear membrane in the cadmium and cooper treatment groups (Figures 5c and 5e). There were no significant differences between the two treatments since either copper or cadmium enter the oocyte by passing through the cell membrane of cumulus cells which resulted in severe damage of the cumulus cells. Figures 5c and 5e show the TEM micrographs of the pCOCs after the 44-h culture in M199 medium containing cadmium or copper at the concentration of 10^{-4} M. The results demonstrated an accumulation of metal at the surfaces of the CCs that led to toxicity and damage to the cells and resulted in loss of the nuclear membrane, but this did not affect the oocyte (Figures 5d and 5f).

Comparisons of the effects of cadmium and copper on the pCOCs revealed that at the same concentration, the toxicity of copper caused more damage to the COCs than cadmium. This is consistent with our previous reports (Sanmanee & Areekijseree, 2009, 2010) and demonstrated that the porcine oviduct epithelial cells cultured in M199 medium containing copper concentration at 10^{-4} M experienced more damage than the cells cultured in cadmium at the same concentration. This finding promotes the merit of using a primary cell culture of pCOCs as a routine *in vitro* cytotoxicity test since it is economical and ethically acceptable on a biological study.

Table 1. Concentrations of cadmium and copper ions detected before and after culture.

Concentration	Time (h)	$CdCl_2$ 10^{-4} M	$CuCl_2$ 10^{-4} M
Initial concentrations in medium (mM)	0	0.098±0.002	0.096±0.003
Final concentrations in medium (mM)	44	0.093±0.004	0.096±0.001
Final concentrations in medium after culture COC 20-30 cells (mM)	44	0.088±0.002	0.089±0.004
Metal concentration in COCs (mM)	44	0.005±0.003	0.007±0.003

5. Conclusions

pCOCs can be classified into four types: intact cumulus cell layers, multi-cumulus cell layers, partial cumulus cell layers, and completely denuded oocyte. The intact and multi-cumulus cell layer oocytes, when cultured in M199 supplemented with 10% HTFCS and hormones, can be efficiently used instead of an animal model for an *in vitro* cytotoxicity assay. The results in this study shed light on the potential of pCOCs as a primary cell culture for cell technology and medical applications.

Acknowledgements

This research was funded by a grant from Silpakorn University Research & Development Institute (SURDI) and Faculty of Science, Silpakorn University, Thailand. We are grateful to Miss Nualanong Narkkong (Central Instruments Center, Faculty of Science, Mahasarakham University) for her assistance in the TEM and SEM technique.

References

Ahmed, M. K., Baki, M. A., Kundu, G. K., Islam, M. S., Islam, M. M., & Hossain, M. M. (2016). Human health risks from heavy metals in fish of Buriganga River, Bangladesh. *Springerplus*, *5*(1). doi:10.1186/s40064-016-3357-0

Aprioku, J. S., Maxwell, B., & Ijomah, M. A. (2014). Toxicological effects of cadmium during pregnancy in Wistar albino rats. *Toxicology and Environmental Health Sciences*, *6*(1), 16-24.

Areekijseree, M., & Chuen-Im, T. (2012). Effects of porcine follicle stimulating hormone, luteinizing hormone and estradiol supplementation in culture medium on ultrastructures of porcine cumulus oocyte complexes (pCOCs). *Micron*, *43*, 251–257. doi:10.1016/j.micron.2011.08.007

Areekijseree, M., & Vejaratpimol, R. (2006). In vivo and in vitro porcine oviductal epithelial cells, cumulus cell complexes and CCs study: A scanning electron microscopy and inverted microscopy study. *Micron, 37*, 707–716. doi:10.1016/j.micron.2011.08.007

Areekijseree, M., & Veerapraditsin, T. (2008). Characterization of porcine oviductal epithelial cells, cumulus cells and granulosa cells-conditioned medium and their ability to acrosome reaction on frozen bovine spermatozoa. *Micron*, *39*, 160–67. doi:10.1016/j.micron.2006.12.008

Bertin, G., & Averbeck, D. (2006). Cadmium: cellular effects, modifications of biomolecules, modulation of DNA repair and genotoxic consequences (a review). *Biochimie, 88,* 1549–1559. doi:10.1016/j.biochi.2006.10.001

Blazka, M. E., & Shaikh, Z. A. (1991). Differences in cadmium and mercury uptakes by hepatocytes: role of calcium channels. *Toxicology Applied Pharmacology*, *110*, 355–363. doi:10.1016/S0041-008X(05)80018-3

Breckle, S. W., & Kahle, H. (1991). Ecological geobotany/ autecology and ecotoxicology. In H. D. Behnke (Ed.), *Progress in botany vol. 52* (pp. 391–406). Berlin, Germany: Springer-Verlag.

Clayton, G. D., & Clayton, F. E. (1981). *Patty's industrial hygiene and toxicology* (3rd ed.). New York, NY: John Wiley and Sons.

Devi, S. R., & Prasad, M. N. V. (1999). Membrane lipid alterations in heavy metal exposed plants. In M. N. V. Prasad & J. Hagemeyer (Eds.), *Heavy metal stress in plants. From molecules to ecosystems* (pp. 127-145). Berlin, Germany: Springer-Verlag Berlin Heidelberg.

Dobrzański, Z., Kołacz, R., & Bodak, E. (1996). Heavy metals in animal environment. *Medycyna. Weterynaryjna*, *52*, 570–574.

Fieten, H., Leegwater, P. A., Watson, A. L., & Rothuizen, J. (2012). Canine models of copper toxicosis for understanding mammalian copper metabolism. *Mammalian Genome*, *23*, 62–75. doi:10.1007/s00335-011-9378-7

Georgopoulos, P. G., Roy, A., Yonone-Lioy, M. J., Opiekun, R. E., & Lioy, P. J. (2001). Environmental copper: environmental dynamics and human exposure issues. *Journal of toxicology and environmental health part B*, *4*(4), 341-394. doi:10.1080/10937401753146207

Hodson, P. V., Borgmann, U., & Shear, H. (1979). Toxicity of Copper to Aquatic Biota. In J. O. Nriagu (Ed.), *Copper in the Environment. Part 2: Health Effect* (pp. 307-372). New York, NY: Wiley and Sons.

Kitiyanant, Y., Tocharus, C., Areekijseree, M., & Pavasuthipaisit, K. (1995). Swamp buffalo oocytes from trans-vaginal ultrasound-guided aspiration fertilized and co-cultured *in vitro* with bovine oviductal epithelial cells. *Theriogenology*, *43*, 250. doi:10.1016/0093-69 1X(95)92404-W

Kuo, C. C., & Hess, P. (1993). Ion permeation through the L-type Ca^{2+} channel in rat phaeochromocytoma cells: two sets of ion binding sites in the pore. *Physiology*, *466*, 629–655. doi:10.1113/jphysiol.1993.sp019738

Leoni, G., Bogliolo, L., Deiana, G., Berlinguer, F., Rosati, I., Pintus, P. P., . . . Naitana, S. (2002). Influence of cadmium exposure on *in vitro* ovine gamete dysfunction. *Reproductive Toxicology*, *16*, 371–77. doi: 10.1016/S0890-6238(02)00040-0

Magnusson, C. (1980). Role of cumulus cells for rat oocytes maturation and metabolism. *Gamete Research*, *3*, 133–140. doi:10.1002/mrd.1120030205

Mori, T., Amano, T., & Shimizu, H. (2000). Roles of gap junctional communication of cumulus cells in cytoplasmic maturation of porcine oocytes cultured *in vitro*. *Biology of. Reproduction*, *62*, 913–919. doi:10.1095/biolreprod62.4.913

Nad, P., Massanyi, P., Skalicka, M., Korenekova, B., Ciganka, V., & Almasiova, V. (2007). The effect of cadmium in combination with zinc and selenium on ovarian structure in Japanese quails. *Journal of environmental science and health. Part A, Toxic/ hazardous substances & environmental engineering*, *42*, 2017–2022. doi:10.1080/10934520701629716

Nordberg, G. F., Herber, R. F. M., & Alessio, L. (1992). *Cadmium in the Human Environment: Toxicity and Carcinogenicity*. Lyon, France: International Agency for Research on Cancer.

Portillo, F. (2000). Regulation of plasma membrane H+ -ATPase in fungi and plants. *Biochimica et Biophysica Acta*, *1469*, 31–42.

Prozialeck, W. C., Edwards, J. R., Nebert, D. W., Woods, J. M., Barchowsky, A., & Atchison, W. D. (2008). The vascular system as a target of metal toxicity. *Toxicological Sciences*, *102*, 207-218. doi:10.1093/toxsci/kfm263

Salt, D. E., Smith, R. D., & Raskin, I. (1998). Phytoremediation. *Annual Review of Plant Physiology and Plant Molecular Biology*, *49*, 643–668. doi:10.1146/annurev.arplant.49.1.643

Sanmanee, N., & Areekijseree, M. (2009). *In vitro* toxicology assessment of cadmium bioavailability on primary porcine oviductal epithelial cells. *Environmental Toxicology and Pharmacology*, *27*, 84–89. doi:10.1016/j.etap.2008.08.012

Sanmanee, N., & Areekijseree, M. (2010). The effects of fulvic acid on copper bioavailability to porcine oviductal epithelial cells. *Biological Trace Element Research*, *135*, 162–173. doi:10.1007/s12011-009-8508-5

Satarug, S., & Moore, M. R. (2004). Adverse health effects of chronic exposure to low-level cadmium in foodstuffs and cigarette smoke. *Environmental Health Perspectives*, *112*, 1099–1103. doi:10.1289/ehp.6751

Schaller, G. E., & Sussman, M. R. (1988). Phosphorylation of the plasma membrane H+-ATPase of oat roots by a calcium-stimulated protein kinase. *Planta*, *173*, 509–551. doi:10.1007/BF00958964

Staigmiller, R. B., & Moor, R. M. (1984). Effect of follicle cells on the maturation and developmental competence of ovine oocytes matured outside the follicle. *Gamete Research*, *9*, 221–229. doi:10.1002/mrd.1120090211

Stawarz, R., Zakrzewski, M., Marenčík, A., & Hraška, Š. (2003). Heavy metal concentration in the toad *Bufo Bufo* from a region of Mochovce, Slovakia. *Ekologia Bratislava*, *22*, 292–297.

Templeton, D. M., & Liu, Y. (2010). Multiple roles of cadmium in cell death and survival. *Chemico-Biological Interactions*, *188*, 267-275. doi:10.1016/j.cbi.2010.03.040

Thompson, J., & Bannigan, J. (2008). Cadmium: toxic effects on the reproductive systemand the embryo. *Reproductive Toxicology*, *25*(3), 304–315. doi:10.1016/j.reprotox.2008.02.001

Toraason, M., & Foulkes, E. C. (1984). Interaction between calcium and cadmium in the 1, 25-dihydroxyvitamin D3 stimulated rat duodenum. *Toxicology Applied Pharmacology*, *75*(1), 98–104. doi:10.1016/0041-008X(84)90080-2

Verbost, P. M., Flik, G., Lock, R. A. C., & Wendelaar Bonga, S. E. (1988). Cadmium inhibits plasma membrane calcium transport. *The Journal of Membrane Biology*, *102*, 97–104. doi:10.1007/BF01870448

Wang, S. H., Shih, Y. L., Lee, C. C., Chen, W. L., Lin, C. J., Lin, Y. S., . . . Shih, C. M. (2009). The role of endoplasmic reticulum in cadmium-induced mesangial cell apoptosis. *Chemico-Biological Interactions*, *181*, 45–51. doi:10.1016/j.cbi.2009.05.004

Washko, P., & Cousins, R. J. (1977). Role of dietary calcium and calcium binding protein in cadmium toxicity in rats. *Journal of Nutrition*, *107*, 920–928.

Watanabe, T., & Endo, A. (1997). Cytogenetic effects of cadmium on unfertilized oocytes in short-term zinc deficiency in hamsters. *Mutation Research, 395*, 113–118. doi:10.1016/S1383-5718(97)00151-4

Williams, P. L., James, R. C., & Roberts, S. M. (2000). *Principles of Toxicology: Environmental and Industrial Applications* (2nd ed.). New York, NY: Wiley and Sons.

World Health Organization. (1992). *IPCS Environmental Health Criteria 134: Cadmium*. Geneva, Switzerland: Author.

Wright, D. A., & Welbourn, P. (2002). *Environmental Toxicology (Cambridge Environmental Chemistry Series).* Cambridge, England: Cambridge University Press.