EVALUATION OF AN ALTERNATIVE *IN VITRO* TESTICULAR CELL CO-CULTURE MODEL FOR MALE REPRODUCTIVE TOXICITY TESTING

by

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(Under the Direction of Xiaozhong Yu)

ABSTRACT

The development of *in vitro* models for detecting testicular toxicity to serve as an animal-friendly, cost-effective and simple alternative is important. Different *in vitro* primary cells co-culture models have been reported, but none of them have been widely applied due to the complexity of the cell isolation procedure, animal difference and the poor ability to replicate the tests. As a continuation of previous studies, a new co-culture model using cell lines from rat testis was established to reduce the usage of animals. The ability of the model in discriminating testicular toxicants was also explored by using neutral red uptake assay. I also treated each single cell type to compare with the co-culture and identify chemicals' sensitive cell types. Specifically, the cytotoxicity of 32 compounds was examined in co-culture for 24 h and 48 h. I observed that this model was able to distinguish testicular toxic and non-toxic chemicals, and more importantly, prioritize the chemicals at high risk by cluster analysis. The linear regression and Pearson's correlation results from the co-culture model after 48 h treatment had the highest correlation with *in vivo* reproductive lowest observed adverse effect level. These results

demonstrate that our *in vitro* co-culture model may be a useful tool in screening testicular toxicants.

INDEX WORDS: Male reproductive toxicity, co-culture model, NR dye uptake, testicular toxicity

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Reproductive disorders caused by exposure to environmental chemicals are a prominent health issue worldwide (Allard *et al.*, 2013). Millions of couples are infertile, and the complications related to the male alone or in combination with the females accounts for up to 75% of the cases (Mathur and D'Cruz, 2011). Male fertility depends on fully functional spermatozoa production through the complex process of spermatogenesis in testes. The male spermatogenesis process can be affected by several categories of chemicals, such as heavy metals, chemotherapeutic agents, antibiotics, solvents, pesticides and plasticizers (Reis *et al.*, 2015). These toxicants can alter sperm production by decreasing sperm count, increasing the number of abnormal sperm, or damaging sperm DNA. Therefore, there is an urgent need to evaluate male testicular toxicity in a variety of environmental chemicals.

In vivo animal evaluation is the traditional way to assess the toxicity of chemicals. While this approach is effective, it is costly, time-consuming and animal-intensive. Reproductive toxicity tests, which include testicular tests in animals, in compliance with Registration, Evaluation, Authorization and Restriction of Chemicals (REACH), would require up to 3,200 animals per substance tested. Applied to thousands of chemicals, the number of animals required would be huge. Thus, *in vivo* assays are not feasible for testing a large number of chemicals for detecting testicular toxicity (Balls, 1994; Balls and Fentem, 1999; Bremer, 2008). Additionally, the Cosmetics Directive of the European Union (EU) eliminated animals from evaluations of reproductive toxicity for cosmetic products (Martin *et al.*, 2011). The 3R paradigm (replacement,

reduction and refinement of animal experimentation) also calls for the development of alternative methods (Tornqvist *et al.*, 2014). Therefore, there is an urgent need for alternative testing strategies for testicular toxicity (Bremer, 2008).

There are two reasons that developing *in vitro* methods may be a good solution. Firstly, testes are among the most sensitive organs to chemical damage (Parks Saldutti *et al.*, 2013). This sensitivity to damage can be easily recorded *in vitro*. Secondly, *in vitro*-cell based approaches can predict whole-animal toxicity. For many chemicals, toxicity at the cellular level is ultimately manifested at the organ level, or affects the viability of the whole organism (Zapor, 2004). Many chemicals exert cytotoxic effects on cell cultures at concentrations that would be toxic to animals. The mechanism of organ-specific toxicity is ultimately determined at the cellular level, which is due to direct or indirect molecular interactions with cellular components (Kavlock and Dix, 2010). The function of testes is to produce male gametes and male sexual hormones. Spermatogenesis describes and includes all the processes involved in the production of gametes. Important cell types involved in spermatogenesis include germ cells and Sertoli cells. Sertoli cells coordinate the spermatogenic process functionally and physically. Each individual Sertoli cell is in morphological and functional contact with a defined number of sperm. A higher number of Sertoli cells are correlated with increased sperm production in normal conditions (Johnson *et al.*, 1994).

Steroidogenesis, a key function of testes, exerts indirect control over spermatogenesis. The most important regulators of steroidogenesis are Leydig cells. Leydig cells produce and secrete testosterone to regulate the spermatogenesis. Leydig cells are regulated by Sertoli cells through growth factors and differentiation factors (Hareng *et al.*, 2005). Thus, Sertoli cells, Leydig cells and germ cells are closely related to each other in spermatogenesis in testes.

Thus, various *in vitro* methods that mimic testes physiology have emerged. Based on the physiology of testes, three kinds of cells (germ cells, Leydig cells and Sertoli cells) were shown to be sensitive to chemicals' treatment. Thus, the available models focused on these three cell types. These *in vitro* models demonstrated some *in vivo* responses, such as steroidogenesis and part of the spermatogenesis. Some of the currently available *in vitro* models are listed in Table 1.1.

Table 1.1. Single and co-culture cell models

Table 1.1.A Leydig cell models

Model types/Species	**Application/characteristics**
MLTC-1 cells (Mouse)	Effects of low concentrations of DEHP and MEHP on steroidogenesis observed. P450scc, P450c17 and 3βHSD under study showed increased expression following exposure to DEHP or MEHP. Testosterone secretions were stimulated after exposure (Chen *et al.*, 2013; Hu *et al.*, 2013).
MA-10 cells (Rat)	MEHP affected the steroidogenic pathway and cAMP production (Zhou *et al.*, 2013).
BLTK1 cells (Rat)	Possessing a fully functional steroidogenic pathway that produces low basal levels of testosterone and expresses all the necessary steroidogenic enzymes including Star, Cyp11a1, Cyp17a1, Hsd3b1, Hsd17b3 and Srd5a1. This model can also be used to elucidate the different mechanisms underlying toxicant-mediated disruption of steroid genesis (Forgacs *et al.*, 2012).

For the Leydig cell culture models, three commonly used Leydig cell lines were listed (MA-10 Leydig tumor cells, MLTC-1 cells and BLTK1 cells). Most of the studies focused on the mechanisms of chemicals about how they exert toxicity, including the changes in steroidogenesis pathways and testosterone levels. The expression level of genes including Star, Cyp11a1, Cyp17a1, Hsd3b1, Hsd17b3, Srd5a1 and cAMP genes in steroidogenesis were often investigated. The applications of Leydig cells in the screening of testicular toxicants were not found in this literature search.

Table 1.1.B Sertoli cell models

Model types/Species	**Application/characteristics**
TM4 cells (Rat)	The relationship between PRPS2 (phosphoribosylpyrophosphate synthetases 2), a subset of PRS (phosphoribosylpyrophosphate synthetases), and Sertoli-cell- only syndrome were investigated on TM4 Sertoli cells. It is found that PRPS2 overexpression significantly inhibited cell apoptosis and promoted cell cycle transition in TM4 Sertoli cells. Moreover, the results revealed that PRPS2 overexpression inhibited cell apoptosis via p53/Bcl-2/caspase-9/caspase-3/caspase-6/caspase-7 signaling pathway (Lei *et al.*, 2015).
15P-1 (Rat)	Estrogen insufficiency or overexposure will lead to impaired spermatogenesis and infertility, suggesting that maintenance of a delicate balance between androgens and estrogens is critical for normal testicular function. Thus, whether the estrogen receptor 1 (Esr1) express on Sertoli cells was investigated. It was found that ligand-dependent nuclear ESr1 was present in mouse Sertoli cells and mediates a classical genomic action of estrogens (Lin *et al.*, 2014).

Primary cells (Mouse)	The organismal roles of the ubiquitously expressed class I PI3K isoform p110β was investigated in the paper. It was found that p110β leads to embryonic lethality in a substantial fraction of mice. It was also found that p110β inactivation dampening expression of the Sertoli Cell-specific androgen receptor target gene Rhox5, a homeobox gene critical for spermatogenesis (Guillermet-Guibert *et al.*, 2015).

For the Sertoli cell models, the available studies often focused on how chemicals exert toxicity to Sertoli cells, such as how low concentrations of bisphenol A can affect the proliferation of Sertoli cells (Ge *et al.*, 2014); the pathways involved in the apoptosis of Sertoli cells by 4-n-nonyphenol (Liu *et al.*, 2015). In the 2011 testicular toxicity workshop (http://www.hesiglobal.org/files/public/Committee%20Presentations/DART/Testicular%20Tox%20Wksp/4Sasaki.pdf), Sertoli cells were discussed as a model to screen testicular toxicants. The TM4 cells and 15P-1 cells were treated with testicular toxicants, and the chemicals' toxicities on TM4 cells and 15P-1 were correlated with the chemicals' testicular toxicity to rats. The biggest problem was that the relative toxicity of these compounds to Sertoli cells did not correlate well with *in vivo* outcomes (Parks Saldutti *et al*., 2013).

Table 1.1.C Sertoli cell-germ cell co-culture models

Model types/Species	Application/characteristics
Primary Sertoli and germ cells co-culture (Rat)	The in vitro 3D-SGC system was employed to examine the effects of cadmium (Cd) and phthalates. The time- and dose-dependent effects of Cd and phthalates on morphological alteration and cell viability were investigated. Microarray analysis was also conducted to investigate the genes affected by phthalates. The results suggested that Cd and developmentally toxic phthalates (DPP, DEHP, DBP and BBP) can induce testicular toxicity, and disrupt steroidogenesis and testosterone-related gene expression. The developmentally non-toxic phthalates tested (DOTP, DMP and DEP) did not display toxicity, supporting this model as an efficient, highly reproducible alternative in assessing reproductive toxicity of environmental agents (Yu *et al.*, 2009; Yu *et al.*, 2005).
Seminiferous tubules from testes of 7-day-old mice (Mouse)	Intratubular cells were cultured in the upper layer of the three-dimensional agar culture system (SACS) in RPMI medium supplemented with fetal calf serum (FCS). The lower layer of the SACS contained only RPMI medium supplemented with FCS. Results revealed that it is possible to induce mouse testicular pre-meiotic germ cell expansion and induce their differentiation to spermatozoa in SACS. The spermatozoa showed normal morphology and contained acrosomes (Abu Elhija *et al.*, 2012).
Primary cultures of testes (Rat)	Relaxin and its receptor RXFP1 are co-expressed in Sertoli cells, and relaxin can stimulate proliferation of Sertoli cells. In this study, the role of relaxin in spermatogenesis was investigated using a short-term culture of testicular cells of the rat that allowed differentiation of spermatogonia to spermatids. Sertoli, germ and peritubular myoid cells were the predominant cell types in the culture. It was found that Relaxin may affect spermatogenesis by modulating spermatogonial self-renewal and favoring cell contact (Pimenta *et al.*, 2015).
Primary seminiferous tubules from 28-day-old rats (Rat)	The meiotic progression of spermatocytes was found to be dependent on Sertoli cell-activated mitogen-activated protein kinases (MAPKs) was investigated by immunostaining (Godet *et al.*, 2008).

Primary seminiferous tubules from 23-day-old rats (Rat)	The effects of tributyltin chloride (TBTC) on Sertoli-germ cell co-culture in ex-vivo and in the testicular tissue in-vivo conditions were investigated. The data indicated the toxic manifestations of TBTC on the male reproductive system and the mechanisms involved (Mitra *et al.*, 2013).
Seminiferous tubule cells (Mouse)	The co-culture is composed of the constituents of testicular stem cell niche: Sertoli cells, peritubular myoid cells and spermatogonia. The formation of cord-like structures was found after approximately 2–4 weeks of culture a spontaneous was monitored. The spontaneous reconstruction of testicular cellular microenvironments was established (Makela *et al.*, 2014).
Xenografts	The fetal rat, mouse, and human testes were xenografted into immunodeficient hosts. The hosts were treated with phthalate esters doses for three continuous days. It was found that rats, mouse and human had multinucleated germ cells formation. Rats showed suppressed steroidogenesis (Heger *et al.*, 2012).

Thus, researchers proposed that due to the complexity of testes, single cell models should not replicate testes well; co-culture models, including more cell types, may be a better solution. Much research focused on co-cultures of Sertoli cells and germ cells as a solution because of their close relationship in spermatogenesis. Several studies focused on how to improve the culture conditions to increase the cell viability of primary cells and the possibility to differentiate germ cells (Gray and Beamand, 1984; Stummann *et al.*, 2008). Other studies found that mixed cell models improved cellular interactions and cell viability (Lee *et al*., 2006). Xenografts were effective in mechanism studies of the toxicity of chemicals, and it can resemble *in vivo* situation better than co-cultures. Different toxicants were treated to co-culture cells to explore the mechanisms of their toxicities (Stummann *et al*., 2008). Our group treated the Sertoli/germ cell co-culture isolated from 5-day-old rats with phthalates and found that this model can successfully distinguish testicular toxic phthalates from non-toxic phthalates (Yu *et al.*, 2009).

By reviewing the available *in vitro* co-culture models, the thesis discovered some limitations. Firstly, these models only include Sertoli cell and round germ cells, not Leydig cells, which are very important for indirect regulation of spermatogenesis through androgen regulation; secondly, the cell viability of primary cells was constantly decreasing in primary cell models. Most importantly, primary cell models require newborn animals to isolate cells, which requires lots of animals and a large amount of money. The xenografts are effective in mechanism studies of chemical which resembles testis *in vivo.* Considering the testis isolation procedure and special host animals' requirement, the resources needed for screening large numbers of chemicals will be huge. Therefore, appropriate *in vitro* models, addressing these limitations, need to be developed to generate a sufficient testing strategy to overcome these weaknesses.

Objectives outline of thesis research

A literature review presented the need for identifying testicular toxicants; the limitations of *in vivo* models, previously published *in vitro* models and future directions in improving these models. In particular, single cell models and co-culture models and their application are discussed in detail. Pointing out the current status of these models provided us with some future directions.

The purpose of this research is to examine the effectiveness of the *in vitro* co-culture model in distinguishing testicular toxicants. Three kinds of important cells in testes, Spermatogonia A cells, Leydig cells and Sertoli cells, were shown to be most sensitive to chemical treatment. This model includes all three kinds of cells that were most important in testis development and function and focuses on identifying the toxicity of chemicals of the early stages of testis development of rats. We hypothesize that this model may be effective to recognize

environmental chemicals' toxicity to testes and prioritize chemicals of potential high safety risk. This thesis discusses the effects of 32 chemicals to the cell viability of co-culture model and single cell types. The toxicities of chemicals were represented as the half maximal inhibitory concentration (IC_{50}) to indicate their toxicities in our system. The comparison between their toxicity *in vitro* (IC_{50}) and *in vivo* (reproductive lowest observed adverse effect level from database) was conducted. Sensitivity, specificity and concordance were calculated (Chapter 2). The results of the research were then summarized and conclusions are discussed in the final chapter (Chapter 3).

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CHAPTER 2

EVALUATION OF AN ALTERNATIVE *IN VITRO* TESTICULAR CELL CO-CULTURE MODEL FOR MALE REPRODUCTIVE TOXICITY TESTING

Introduction

Male fertility depends on fully functional spermatozoa production through spermatogenesis. This complex process may be affected by several categories of chemicals, such as heavy metals, chemotherapeutic agents and antibiotics, solvents, pesticides and plasticizers (Reis *et al*., 2015). These toxicants can alter sperm production by decreasing sperm count, by increasing the number of abnormal sperm or by damaging sperm DNA. Reproductive disorders caused by exposure to these toxicants have been a prominent health issue worldwide (Allard *et al*., 2013). Addressing this problem requires the evaluation of toxicity of many and varied environmental chemicals.

Currently *in vivo* tests for chemical toxicity are resource-intensive, particularly for multi-generation reproductive and prenatal developmental assessment (Balls, 1994; Balls and Fentem, 1999; Bremer, 2008). It has been estimated that 70% of the total cost and 90% of the animals used for compliance with the Registration, Evaluation, Authorization, and Restriction of Chemicals (REACH) regulation will be due to reproductive toxicity testing (Hartung and Rovida, 2009). Thus, current toxicity testing approaches based on whole animals are unlikely to screen all chemicals for hazard identification in a timely and cost-effective manner. Policy directives such as the Cosmetics Directive of the European Union (EU) called for the elimination

of animals for evaluating reproductive toxicity by 2013 for cosmetic products and the development of alternative methods for safety evaluation (Martin *et al*., 2011). Additionally, animal welfare concerns expressed by media and non-governmental organizations have also pushed the need to find *in vitro* methods as replacement. Recent increasing restrictions on animal studies have persuaded researchers to seek *in vitro* model alternatives to reduce the use of experimental animals (Prati *et al.*, 2002).

In 2007, the U.S. Environmental Protection Agency (EPA) launched a large-scale program (ToxCast) to investigate high-throughput, *in vitro* assays to prioritize substances for further in-depth toxicological evaluation, identify mechanisms of action for further investigation and develop predictive models for *in vivo* biological response (Houck *et al.*, 2009). The main focus of the ToxCast program is to generate different bioactivity profiles for chemicals' *in vitro* biological activities and to correlate with *in vivo* toxicity data from massive *in vivo* animal studies. After generating the predictive model, researchers can use it to detect potential toxicants for which sufficient toxicity data are not available (Bremer *et al.*, 2005). However, of all the models in the ToxCast program, none are specifically designed for detecting male reproductive system toxicity. It is therefore important to construct a reliable model to fill this gap. Various *in vitro* models have been published, including Sertoli cell-enriched cultures model (Gray 1986; Hadley *et al.*, 1985), Leydig cell-enriched cultures model (Yang *et al.*, 2003), Leydig/Sertoli cell culture model (Bilinska 1989) and Sertoli/germ cells model (Chapin *et al.*, 2013a). Application of these models has been limited, due to the complexity of cell isolation procedure, species differences and the poor ability of these *in vitro* culture systems to replicate the complex biochemical, molecular and functional interactions observed *in vivo* (Yu *et al*., 2005).

Our group previously developed an *in vitro* three-dimensional Sertoli cell/gonocyte co-culture model (3D-SGC) with extracellular matrix (ECM) overlay (Wegner 2014; Wegner *et al.*, 2013; Wegner *et al.*, 2015; Yu *et al.*, 2008; Yu *et al.*, 2009; Yu *et al.*, 2005). The *in vitro* 3D-SGC system was employed to examine the effects of cadmium (Cd) and phthalates. Our lab investigated the time and dose dependent effect of Cd and phthalates on morphological alteration and cell viability. Microarray analysis was also conducted to investigate the genes affected by phthalates. The results suggested that Cd and testicular toxic phthalates could induce testicular toxicity. Toxic phthalates (DPP, DBP, BBP and DEHP) can disrupt steroidogenesis and testosterone-related gene expression. Non-toxic phthalates (DEP, DMP and DOTP) did not display reproductive toxicity. It is therefore shown to be a simple, efficient and highly reproducible alternative in assessing reproductive toxicity of environmental agents. However, some limitations still exist. The co-culture cells were primary cells isolated from five-day old rats' testes. The process requires lots of money, effort and animals. Also, primary cells are not of unlimited supply and need repeated isolation from rats to screen large numbers of chemicals.

The objective of this study was to construct a co-culture system by using cell lines instead of primary cells, reducing the use of animals. The same three cell types, spermatogonia A, Leydig cells and Sertoli cells, were included. By using spermatogonia A cells, our model specifically focused on early development of testis in rats. We hypothesize that this model may be effective in identifying testicular toxic chemicals *in vitro*. To test our hypothesis, 32 chemicals were tested in our co-culture model. We also treated each single cell type to compare with the co-culture. The correlation of chemicals' toxicity *in vivo* and *in vitro* was then assessed. We observed that this co-culture model was able to distinguish testicular toxicants and had

moderate correlation with *in vivo* outcomes. Thus, our *in vitro* co-culture model may be a useful tool for screening environmental chemicals for testicular toxicity.

Materials and methods

Chemicals. Dulbecco's modified Eagle's medium (DMEM), Nu-Serum Growth Medium Supplement (Nu-serum), fetal bovine serum (FBS), antibiotic-antimycotic (penicillin G sodium, streptomycin sulfate), 0.25% Trypsin-0.02% EDTA solution, 0.4% trypan blue stain, Dulbecco's Modified Eagle's Medium/Nutrient F-12 Ham (DME-F12), neutral red solution (0.33%), Hanks' balanced salt solution, Dulbecco's phosphate buffered Saline (PBS) and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (St. Louis, MO); glacial acetic acid (100%) was obtained from from Merck (Darmstadt, Germany); 100% ethanol, carboxyl-DCFDA (5-(and-6)-Carboxy-2', 7'-Dichlorofluorescein Diacetate) mixed isomer was obtained from Life Technologies (Gaithersburg, MD). 2-methoxyethanol (ME), 2,2-Bis(4-hydroxy-3-methylphenyl)propane (BPA), boric acid (BA), tri-(2-chloroethyl)phosphate (TCEP), dioctyl phthalate (DEHP), mono-(2-ethylhexyl) phthalate (MEHP), β-Estradiol (ES), 4,4'-(hexafluoroisopropylidene) diphenyl (BPAF), 2,4,4′-trichloro-2′-methoxydiphenyl ether (TCS), 4,4′-sulfonyldiphenol (BPS), benzophenone-3,3′,4,4′-tetracarboxylic dianhydride (BEN), saccharin sodium (SAC), 3,3',5,5'-tetrabromobisphenol A (TBBPA), diethyl phthalate (DEP), dimethyl phthalate (DMP), hydroxyurea (HU), valproic acid sodium salt (VPA), zearalenone (ZEA), cadmium chloride ($CdCl_2$), dioctyl terephthalate (DEHP), benzyl butyl phthalate (BBP) and diphenyl phthalate (DPP) were obtained from Sigma-Aldrich (St. Louis, MO). Tricresyl phosphate (TCP), vinclozolin (VIN), diethylstilbestrol (DES), heptachlor (HEX)

hexachlorophene (HEP) were obtained from Fisher Scientific (Gaithersburg, MD). Diazinon (DIA), parathion (PARA) and chlorpyrifos (CHL) were obtained from Chem Service (West Chester, PA). Arsenic (As), as sodium arsenite, was obtained from GFS Chemicals (Powell, OH).

Cell culture. Leydig cells and Sertoli cells (rats) were purchased from ATCC. The passages of Leydig cells and Sertoli cells we used were between the third passages to the forty passages. Spermatogonia A cells which are spermatogonial stem cells were generously donated by Marie-Claude Hofmann (The University of Texas MD Anderson Cancer Center) (Hofmann *et al.*, 2005). The spermatogonia A cells were used between the twelve passages to the forty passages. Leydig cells and Sertoli cells were cultured in DME/F12 medium supplemented with 1% streptomycin and penicillin, 5% Horse Serum, and 2.5% FBS, and maintained at 37˚C in a humidified chamber with 95%/5% air/CO_2. Spermatogonia A cells were cultured in DMEM/High Glucose medium supplemented with 5% FBS and were maintained at 33˚C in a humidified chamber with 95%/5% air/CO_2 (Hofmann et al., 2005). The aim of the co-culture was to mimic the response of testes of rats in early stages. Based on the percentages of these three cell types in 5-day old rats, the co-culture of the three cell types was prepared at the percentage of 80% spermatogonia A cells, 15% Sertoli cells, and 5% Leydig cells, and cultured in DMEM/High Glucose medium at 33˚C, supplemented with 5% FBS with a humidified atmosphere in a humidified chamber with 95%/5% air/CO_2.

Assessment of cell morphology. Before and following chemical treatment, cellular morphology was examined with a Nikon inverted microscope with phase-contrast optics to assess general appearance. The images were randomly captured using a Nikon digital camera (Nikon, Japan), and processed using Illustrator CS6 (Adobe, Seattle, WA).

Neutral red (NR) dye uptake assay. All cultures were viewed under an inverted microscope to assess the general appearance of cells. Cell viability was measured through NR dye uptake assay (Sidhu *et al.*, 2006). Cells were seeded to 96-well plates. The density of co-culture cells was $3.5x10^4$ per well. The density for single cell types was $2.0\ x10^4$ per well. The density of the cells was determined to achieve 100% confluence for co-culture and 70-80% confluence for single cell types after overnight. Cells were treated with different chemicals at 4 concentrations with 5 replicates for each concentration. After 24 h or 48 h, the medium was replaced with a medium containing NR dye (50μg/cm^3, 200μl per well). After incubation for 3 h, supernatants were removed and the cultures washed with PBS twice. The cells were then fixed with 200 μl 1% acetic acid-50% ethanol-50% distilled water. The plates were shaken for 10 min at room temperature. The absorption was measured at 540nm. After deducting blank wells (no cells), cell viability was calculated as a percentage of optical density (OD) readings of treatment divided by control group. The dose-response curve for each chemical was generated based on the cell viabilities.

A literature search in the PubMed database for available cytotoxicity tests was performed to identify the concentration ranges. The highest concentration selected caused no less than 50% decrease of cell viability in the response curves of available cytotoxicity tests. After generating the dose-response curve in my co-culture system, the concentration range was adjusted and until 50% decrease in cell viability was achieved. But within our system, the highest concentrations tested were 50 mM for water-soluble chemicals and 5 mM for DMSO-soluble chemicals.

Literature search for chemicals in vivo toxicity. For all chemicals tested, electronic literature searches for their *in vivo* toxicities were performed in the ToxCast database (http://actor.epa.gov/actor/faces/ACToRHome.jsp), the TOXNET data network

(http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB), the National Toxicology Program's reproductive toxicity study abstracts (http://ntp.niehs.nih.gov/testing/types/repro/abstracts/index.html), and the National Pesticide Information Center (http://npic.orst.edu/) using CAS registry numbers and common names. Based on the review of U.S Environmental Protective Agency's office of Pesticide program, reproductive lowest observed adverse effect levels (rLOAEL) of rats were established to indicate chemicals' reproductive toxicities. The endpoints for determining rLOAEL of rats includes but not limited to primary fertility, early offspring survival, offspring weight, longer-term offspring survival and other systemic offspring toxicities.

Calculation of concordance, specificity, and sensitivity. In order to calculate concordance, specificity, and sensitivity, reproductive toxicants *in vivo* were defined here as having achieved an rLOAEL lower than 500 mg/kg/day (Martin *et al*., 2011). Reproductive toxicants *in vitro* are defined here as having achieved a half maximal inhibitory concentration (IC_{50}) ≤ 1,000 μM, which was done in previous publications of ToxCast results (Martin *et al.*, 2011). The conversion of IC_{50} to rLOAEL of each chemical was closely related to the molecular weight. Reproductive toxicants were marked as "+" and reproductive non-toxicants were marked as"-" both *in vivo* and *in vitro*. Chemicals with insufficient data in the literature were marked "NA" for "no available information". The concordance statistics is to denote the probability that a randomly selected subject who experienced the outcome will have a higher predicted probability of having the outcome occur compared to a randomly selected subject who did not experience the event (Austin and Steyerberg, 2012). Concordance (%) was calculated from the formula 100 × (the proportion of chemical results from the NR dye uptake assay that matched the results from the literature; both positive or negative) (Kligerman *et al.*, 2014).

Sensitivity measures the proportion of positives that are correctly identified as such. Sensitivity (%) was calculated from the formula 100 × (the proportion of chemicals with a positive result in NR dye uptake assay that were positive in the literature). Specificity measures the proportion of negatives that are correctly identified as such. Similarly, specificity (%) was derived from the formula 100 × (the proportion of chemicals with negative results in NR dye uptake assay that were negative in the literature). The chemicals with "NA" for *in vivo* rLOAEL were not included in these calculations of concordance, sensitivity, or specificity.

Statistical analysis. Data files of optical density (OD540) were exported into an Excel spreadsheet. Mean OD values, standard deviations (SD), and cell viabilities based on 5 replicates were calculated. Viabilities of cells were calculated as arithmetic mean percentages of control ± SD. The dose-response curves of each chemical to co-culture and single cell types were plotted. Based on the dose-response curve on co-culture cells, IC_{20}, IC_{50}, IC_{84}, and IC_{90} values were calculated using survival analysis and the probit method with StatPlus software (AnalystSoft Inc., Walnut, CA). The relationship between IC_{50} values and rat rLOAEL values was evaluated using linear regression. The Pearson correlation coefficient between IC_{50} values and rLOAEL was calculated using GraphPad 5.0 (GraphPad Software Inc., La Jolla, CA). Benchmark doses (BMD) were calculated using EPA's BMDS version 2.6. The method used was a continuous model with polynomial parameters. Here, a 10% decrease of cell viability compared with controls was selected (Ihekwereme *et al.*, 2014; Yager *et al.*, 2013). Hierarchical clustering analyses were carried out using average linkage and elucidation distance correlation coefficients in MeV software.

Results

Construction of co-culture system using cell lines and overview of morphology. The co-culture model (Fig. 1A) consisted of three cell types: Spermatogonia A cells (Fig. 1B), Leydig cells (Fig. 1C), and Sertoli cells (Fig. 1D). In terms of morphology, spermatogonia A cells and Leydig cells appeared epithelial-like and polygonal with more regular dimensions. Sertoli cells were fibroblastic-like with multipolar and elongated shapes.

Cell viability changes of co-culture induced by 32 chemicals. All chemicals tested are listed in Table 1. The concentration ranges selected for these chemicals were decided based on the procedures described in Methods. The NR uptake assays were conducted for all 32 chemicals and their dose-response curves were generated. In order to show the dose-response curves of all 32 chemicals in an organized way, the chemicals were arranged into four groups based on their highest concentration tested (Fig. 2). The first group was comprised of chemicals with the highest concentrations tested ≤ 100 μM (Fig. 2A). The second (Fig. 2B) and third (Fig. 2C) group was comprised of 16 chemicals whose tested highest concentrations ranged from 100 μM to 500 μM. Group 2 consisted of all phthalates (Fig. 2B). The fourth group (Fig. 2D) includes chemicals with the highest concentration tested between 5 mM and 50 mM.

In Fig2A, ZEA, Cd and As were most toxic, followed by HEP, DES, BPAF, TBBPA and HEX. In Fig. 2B, a dose-dependent decrease in cell viability was observed following treatment DBP, DEHP, BBP, and DEHP. It was not, however, observed following exposure to DEP, DMP, and DOTP. In the third group (Fig. 2C), ES and BEN did not cause significant decrease in cell viability within the dose range. The toxicity of pesticides in this group at 200 μM, from high to low, was TCS, CHL, DIA, and PARA. Fig. 2D lists all the low-toxicity chemicals whose ordered highest concentrations ranged from 5 mM to 50 mM. By statistical analysis, HU, TCP, VIN,

TCEP, BA and VPA decreased the cell viability significantly at the highest concentration tested. Other chemicals (CYC, ME and SAC) did not cause significant decrease in cell viability across all concentrations.

Testicular toxicants can cause the germ cells detach from Sertoli cells and become rounded (Gray and Beamand, 1984). The morphological changes of co-culture cells under Cd treatment were shown (Fig. 3A-F). In our system, an increase in the number of rounded cells was observed at the low Cd concentration of 5 μM (Fig. 3C), and increased presence at 20 μM Cd (Fig. 3E). Approximately 95% of the cells became rounded with 40 μM Cd (Parks Saldutti *et al*., 2013).

IC_{50} of chemicals. The IC_{50} values of tested chemicals are listed in Table 2. The co-culture cells were first treated with these chemicals. In order to compare single cell cultures and co-culture cells and identify the potential targets in testes, chemicals were then applied to each single cell type. Thus, the IC_{50} values include both co-culture and single cell types at both 24 h and 48 h. For chemicals with the lowest cell viability higher than 50% at the highest concentration, the IC_{50} was calculated out of the dose range and the highest concentrations were assigned to IC_{50} as a substitute (Nakatsu *et al.*, 2007). After comparison, we found that the IC_{50} values at 24 h were generally higher than those at 48 h. For Cd, ZEA, As, HEP, DES, TBBPA, TCS, HEX and CHL, the IC_{50} values were mostly ≤ 100 μM. The IC_{50} values of chemicals in the second and third group (Fig. 2B-C) were mostly ≤ 1,000 μM.

When comparing different cell types, different sensitivities to the same chemical were observed. In general, the lower IC_{50} value, the more sensitive the cell type is. Our results showed that Sertoli cells were more sensitive to BPS, PARA, and TCP. Spermatogonia A cells were

sensitive to Cd, As and HEX. Both Sertoli cells and Leydig cells were sensitive to BPA, BPAF and HEP (Table 2).

Benchmark dose (BMD) of chemicals The BMD values at 48h were listed in Table 3. ZEA, Cd, As, DES, TCS, BPAF, HEX, CHL, BPA, BPS, DPP, DBP and PARA had BMD values less than 20 μM. TBBPA, DIA, DEHP, ES and VIN had BMD values less than 100 μM. For chemicals with cell viability higher than 90% across all concentrations, the highest concentration tested was used as BMD. By comparing BMD values of different cell types, I found that it was quite different across different cell types. The cell type with lower BMD was more sensitive. The result indicated that spermatogonia A cells was more sensitive to ZEA. Sertoli cells were more sensitive to TBBPA and PARA. Leydig cells were more sensitive to HEX and BBP. Both Leydig cells and Sertoli cells seems be sensitive to Cd, As and BPA.

Cluster analysis In order to compare the testicular toxicity among all tested chemicals within our model, a non-supervised two-dimensional hierarchical cluster analysis of the IC_{20}, IC_{50}, IC_{84}, and IC_{90} values of the co-culture after a 48 h treatment was employed (Fig. 4). The figure illustrates the degree to which the chemicals affected cell viability. The chemicals at the top (cluster 1) were the most toxic, and those at the bottom (cluster 3) were the least toxic. Cluster1 included Cd, ZEA, As, HEP, DES, TBBPA, TCS, HEX, and CHL, all of which belong to the first group (Fig. 2A). Cluster 2 included BPAF, HU, BPA, DIA, DPP, BPS, DBP, BBP, BEN, PARA, DEHP, VPA, and ES, mainly chemicals in the second and third groups (Fig. 2B and Fig. 2C). Cluster 3 included DOTP, DEP, DMP, ME, CYC, TCEP and SAC, which belong to the fourth group (Fig. 2D).

Correlation between IC_{50} and rLOAEL. The rLOAEL values listed in Table 4 are an indicator of chemicals' reproductive toxicity *in vivo*. Correlation between IC_{50} and rLOAEL was

established to explore to what extent an *in vitro* IC_{50} could predict an *in vivo* rLOAEL (Table 4) (Martin et al., 2011). Linear regression was established between IC_{50} of co-culture and single cell types and equipotent *in vivo* rLOAEL (Fig 5). The analysis showed that, for 24 h exposures, R^2 values for co-culture, Spermatogonia A cells, Leydig cells and Sertoli cells were 0.43, 0.03, 0.04, and 0.2, respectively. Co-culture had the highest R^2 value compared to all single cell types. For 48 h exposures, R^2 values for co-culture, spermatogonia A cells, Leydig and Sertoli cells were 0.53, 0.1, 0.19, and 0.31. R^2 for co-culture was still the highest among co-culture and single cell types. The R^2 value for 48 h exposures ($p = 0.0009$) was higher than the corresponding R^2 value for 24 h exposures ($p = 0.0109$). Therefore, co-culture at 48 h ($p = 0.0009$) gave the best prediction of rLOAEL values *in vivo* of rats.

Pearson's correlation coefficient was also established to support the results of linear regression. The *r*-values for co-culture, spermatogonia A cells, Leydig cells, and Sertoli cells at 24 h were 0.66, 0.16, 0.21, and 0.48, respectively. For 48 h exposures, the *r*-values were 0.73, 0.31, 0.44, and 0.56. Similarly to R^2 calculations, the co-culture displayed higher *r*-values than single cell types for both 24 h and 48 h exposures. *r*-values for both co-culture and single cell types at 48 h were higher than those of 24 h exposures. In conclusion, linear regression and Pearson's correlation coefficient both showed that co-culture at 48 h had better predictive value for the reproductive toxicity of chemicals *in vivo*.

Concordance, sensitivity, and specificity. Concordance, sensitivity, and specificity were calculated and are listed in Table 5. For *in vivo* evaluation, rLOAEL values were used to represent chemical toxicity. Table 5 shows that BA, TBBPA, DOTP, DMP, DEP, and SAC were all negative *in vivo* and *in vitro*. DIA, DES, Cd, CHL, HU, HEP, HEX, As and ZEA were

positive both *in vivo* and *in vitro*. ME was positive *in vivo*, but negative *in vitro*. For co-culture cells at 48h, TCEP, ME, VIN were negative in co-culture cells, but positive *in vivo*.

The results indicated that the concordance, sensitivity, and specificity were higher at 48 h compared to 24 h across all comparisons. At 48 h, the co-culture, when compared to single cell types, displayed the highest concordance, sensitivity, and specificity values at 79.31%, 85.71%, and 62.5%, respectively. Thus, the false positive rate for the co-culture at 48 h is 14.29% and the false negative rate at 48h is 37.5% at the current conditions. The concordance values for spermatogonia A cells, Leydig cells, and Sertoli cells at 48 h were 73.33%, 73.33%, and 67.74%. Similarly, the sensitivity values for spermatogonia A cells, Leydig cells, and Sertoli cells at 48 h were 80.95%, 80.95%, and 76.19%. Specificity values were 55.56%, 55.60%, and 50.00%, respectively. For 24 h, co-culture still had the highest concordance, sensitivity, and specificity values; concordance values for co-culture, spermatogonia A cells, Leydig cells, and Sertoli cells were 65.63%, 48.57%, 44.44%, and 52.94%. Sensitivity values were 71.43%, 57.14%, 52.38%, and 61.91%. Specificity values were 45.46%, 35.71%, 33.33%, and 38.00%. In summary, the co-culture showed the highest values for concordance, sensitivity, and specificity across all conditions after the 48 h treatment.

Discussion

Continuing our efforts to construct co-culture systems to screen for testicular toxicants, this co-culture system was treated with 32 chemicals in total. Different cell types were then treated with these chemicals. The co-culture and single cell cultures were compared to distinguish a better condition to screen testicular toxic chemicals. Each single cell type is

sensitive to a certain category of chemicals; Leydig cells, which express androgen receptors, are very sensitive to endocrine disruptors. Due to the complexity of testes, the concentrations that chemicals were toxic to single cell types, were not corresponding to the concentrations *in vivo*. It is proposed that single cell types should not be as representative as co-culture cells that can interactively coordinate different cell types and enables a similar physiological condition in testes. The co-culture, enabling the communication between different cell types, should be a better system than single cell types to screen testicular toxic chemicals. Our hypothesis was confirmed by comparing *in vitro* IC_{50} with *in vivo* rLOAEL of rats. The co-culture offered better correlation with rLOAEL than single cell types through linear regression and Pearson's correlation analysis. The r-value of co-culture at 48 h is around 0.7. Considering the limitations of *in vitro* studies, this value is impressive at this condition.

The single cell types were also used to identify chemicals' potential sensitive types. Each of the three cell types plays a very important role in spermatogenesis. The spermatogenesis includes the process of spermatogonia A cells differentiating into spermatozoa. Sertoli cells support spermatogenesis both functionally and physically. Leydig cells secrete testosterone which can indirect regulate the process. Any toxicity on these three types of cells will eventually affect testis function. Single cell culture models can also help to elucidate the underlying mechanisms of toxicants to testes. For example, Leydig cell models were often used to investigate the chemicals' effect on steroidogenesis.

Cytotoxicity test was usually the first step of in *vitro* tests in replacement of animal studies (Ceridono *et al.*, 2012a). The concentrations in cytotoxicity tests that can lead to non-specific alterations in cellular functions may cause effects on organ level and/or death of the organism (Zapor 2004). NR dye uptake assay is one of the most sensitive and reliable

cytotoxicity tests. It has been employed by Environmental Protection Agency to predict the starting dose of acute oral system toxicity and harmful tobacco smoke toxicants. Thus, in our system, we also used NR dye uptake assay to evaluate the toxicity of these chemicals.

The co-culture can distinguish the toxicity of different chemicals, and more importantly, prioritize chemicals at high risk by cluster analysis. By doing NR uptake assay, the dose response curves of each chemical were acquired. It is not feasible to compare the different toxicities of chemicals using the response-curves because of the different concentration range for each chemical. Therefore, the inhibitory concentrations (IC) from 20% to 90% were calculated to indicate the toxicity of each chemical.

The inhibitory concentrations were then imported to non-supervised cluster analysis to distinguish different toxicities of chemicals. Three clusters were identified and defined as very toxic, medium toxic, and least toxic. After comparing with published *in vivo* studies, I found that our results were consistent with rat data. The rLOAEL values of Cd, ZEA, As, DES and HEP in rats were 0.088, 1, 8, 10, and 3 mg/kg/day, respectively, indicating their high toxicity to rats (Martin *et al*., 2011; Seiler and Spielmann, 2011); they were defined as very toxic in our system. DEP, DOTP and DMP, which belong to the least toxic group, are well known as developmentally non-toxic phthalates (Gray *et al.*, 2000; Yu *et al*., 2009). SAC is a sweetener and often used as a negative reference compound in embryo-toxicity tests; it was also not toxic in our model. Thus, cluster analysis can help to predict the toxicity levels of new chemicals based on the cluster the chemical resides in. The toxicity levels of different categories of chemicals were also assessed. For bisphenols, the order of toxicity was: TBBPA > BPAF > BPA > BPS. For pesticides, the order was: As > HEP > TCS > HEX > CHL > PARA > VIN > BA. For phthalates, the order was: DPP > DBP > BBP > DEHP > DMP, DEP, DOTP.

Different cell types displayed different sensitivities to the same chemical by treating each single cell type, as indicated by IC_{50} values. In our system, Leydig cells were more sensitive to DPP, suggesting that DPP probably be an endocrine disruptor (Kumar *et al.*, 2015). The IC_{50} values of BPA, TCP and Cd for Sertoli cells were lower than for other cell types, indicating that Sertoli cells may be their targets. In literature, DPP is shown to be an anti-androgenic compound and reported to interfere with fetal testicular testosterone production in rats, suggesting its possible targets to be Leydig cells (Sung *et al.*, 2003). Cd and BPA were reported to induce testicular injury at the blood-testis barrier (Sertoli cells) to elicit subsequent damage to germ cells, and cause germ cell loss (Helmestam *et al.*, 2014). The primary targets of TCP in testes are Sertoli cells (Chapin *et al.*, 1991). Thus, the results from treating single cell types may provide some hints regarding chemicals' targets *in vivo*.

The chemicals' toxicity was time-dependent. This was indicated by the higher R-values in Pearson correlation coefficient, and R^2 values in linear regression, at 48 h than 24 h. Chemicals were generally more toxic at 48 h than at 24 h for both co-culture and single cell types. We conclude that the treatment time greatly influences the toxicity of chemicals. In future studies, perhaps more time points could be tested to select the optimal treatment time. The best exposure time for each chemical would enable the prediction of plasma concentration in vivo where the toxicity occurs (McKim 2010).

For chemicals with low toxicity, such as DEP, DMP and DOTP, the 50% decrease in cell viability requires very high doses, making it hard to achieve within the current dose range. Thus, IC10 or IC20 could be used to represent the toxicity of these chemicals. In our research, BMD were listed as another reference for chemicals' toxicity. BMD represents an exposure due to a dose of a substance associated with a specified low incidence of risk, generally in the range of

1% to 10%, of a health effect; or the dose associated with a specified measure or change of a biological effect. The BMD of the tested chemicals was correlated with the corresponding rLOAEL using Pearson's correlation coefficient, but the r-value between co-culture and single cell types were low ($r < 0.4$) and quite similar (data not shown). It may be noticed that the sensitive cell types indicated by IC_{50} and BMD were a little different. The discrimination may be due to the definition of IC_{50} and BMD: IC_{50} is 50% decrease in cell viability and BMD is 10% decrease in cell viability. The selection of different points in the non-linear dose-response curves may contribute to the difference.

It may be noticed that the concordance, sensitivity, and specificity of co-culture and single cell types were quite similar. All three were calculated from a positive-negative set, instead of dose-response curves with exact numbers, rendering them less sensitive. Thus, concordance, sensitivity, and specificity cannot distinguish between co-culture and single cell types well. Also, the number of chemicals tested and changes in the selection of tested chemicals would have great influence on the values of concordance, sensitivity, and specificity. Therefore, the calculation of concordance, sensitivity, and specificity only offers a reference value, instead of trying to determine a certain prediction rate for the assay.

By comparing the current model with our previous primary cells co-culture model, we found that both our cell line model and the primary co-culture model distinguished the toxic phthalates (DEHP, DBP, BBP and DPP) from the non-toxic phthalates (DEP, DMP and DOTP). The current cell line model induced higher cell death than previous primary cells model at the same concentrations for Cd treatment.

Although NR dye uptake assay provided valuable information on the potential toxicity of chemicals, limitations still persist. 1) These were preliminary data of only 32 chemicals in

cytotoxicity tests, which require further validation (Bouhifd *et al.*, 2012; Chandler *et al.*, 2011). 2) More endpoints that correlate with different adverse reproductive effects *in vivo* should be assessed, such as sperm DNA defects and fragmentation, epigenetic changes, DNA damage, and even maternal toxicity (Balls and Fentem, 1999; Chandler et al., 2011; Hareng *et al.*, 2005). 3) Some of the tested chemicals exert their toxicity through bio-activation, such as VIN and phthalates. *In vitro* cell models only have partial metabolic abilities. Therefore, it is better to add bio-activation system or test their metabolites in our system to further detect chemicals' toxicity.

The reproductive toxicity of ME is reported *in vivo*, but without effect in our system. ME was reported to induce testicular atrophy and disruption of spermatogenesis via the metabolism of Ethylene glycol monomethyl ether (EGME) (Foster *et al.*, 1984; Foster *et al.*, 1983). It was found that a 4-week administration of ME at relatively low doses, corresponding to 0.08-0.325 mM/kg/day, was sufficient to produce marked testicular atrophy and a decrease in epididymis and prostate weights in a dose-dependent manner (Starek-Swiechowicz *et al.*, 2015). The possible reason may be the necessity of bio-activation for chemicals to induce their toxic effects (van der Laan *et al.*, 2012). It was found that ME undergoes metabolic activation to appropriate methoxyacetic acid (MAA) via EGME (Takei *et al.*, 2010). MAA primarily affects tissues with rapidly dividing cells and high rates of energy metabolism in testes, leading to apoptosis of primary spermatocytes. MAA is associated with changes in the expression of various genes and signaling pathways including oxidative stress in spermatocytes (Bagchi and Waxman, 2008). MAA was also reported to regulate the transcriptional activity of nuclear receptors including androgen receptor to contribute to the testicular toxicity (Bagchi *et al.*, 2009). Thus, the lack of effect of ME in our model may be due to lack of the complete metabolism *in vitro*. In future experiments, MAA could be tested in our co-culture system to test its toxicity in our system.

Besides the toxicities of these chemicals in rats, the toxicities of these chemicals in rabbits were also identified. Cd was given to rabbits at 12 weeks age in dose of 0.08mmol/kg subcutaneously and lead to decreased sperm output of the male rabbits when adults (Foote 1999). DBP were given to rabbits from 400 mg/kg/day *in utero* (GD 15-29); reduction in ejaculated sperm, weight of testes and accessory sex glands were observed (Higuchi *et al.*, 2003). Rabbits were also treated with VIN from gestation day 15 to postnatal week 4 at the concentration of 7.2 and 72 mg/kg/day. The abnormal spermatozoa were two times more likely compared to control group with nuclear and acrosomal defects. The seminiferous tubules were also more frequent ($p < 0.05$) in treatment group (Veeramachaneni *et al.*, 2006).

A vitally important theme in reproductive toxicity studies is the assessment of *in vitro* and *in vivo* models that are predictive for adverse effects in humans exposed to chemicals. Our *in vitro* cell viability data were directly linked to rat rLOAEL. Thus, those chemicals toxic in our system may be toxic to rats. Considering that the prediction rate of animal studies to human toxicity is only 50% -70% (Chapin *et al.*, 2013b; Olson *et al.*, 2000), there is a certain distance to correctly predict toxic chemicals in human. In order to predict the *in vivo* toxicities, the *in vitro* data has to be linked to human or animal plasma concentration, where the toxicity would occur. Other important factors including protein binding, metabolic stability, metabolic activation, metabolites, temporal relationship and compound solubility must also be addressed. Thus, it is unlikely that any individual *in vitro* model is sufficient as a final decision point for toxicity. A series of models which can provide a series of important information in a tiered approach is needed to improve the prediction of *in vitro* assays (McKim, 2010).

In summary, using cell lines to construct a co-culture model, we demonstrated the possibility of using this technique to distinguish testicular toxic from non-toxic chemicals. The

single cell types were also treated with chemicals and compared with the co-culture model. The potential targets of chemicals in testes were implied. Moreover, the highest correlation with *in vivo* data was found in the co-culture model. It is demonstrated that our *in vitro* co-culture model may be a useful alternative for screening testicular toxicants (Ceridono *et al.*, 2012b).

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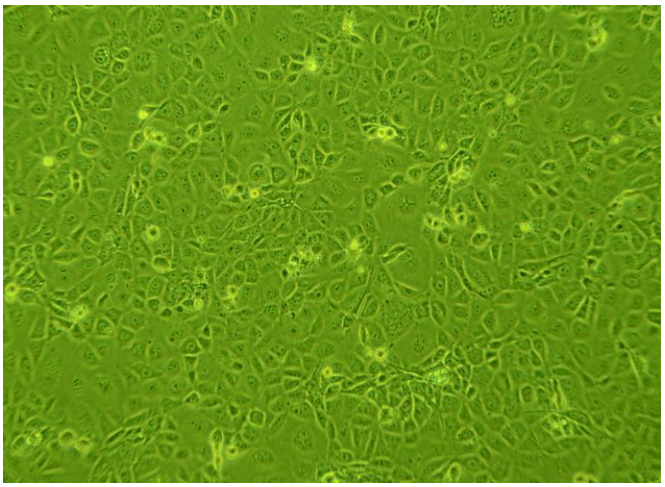
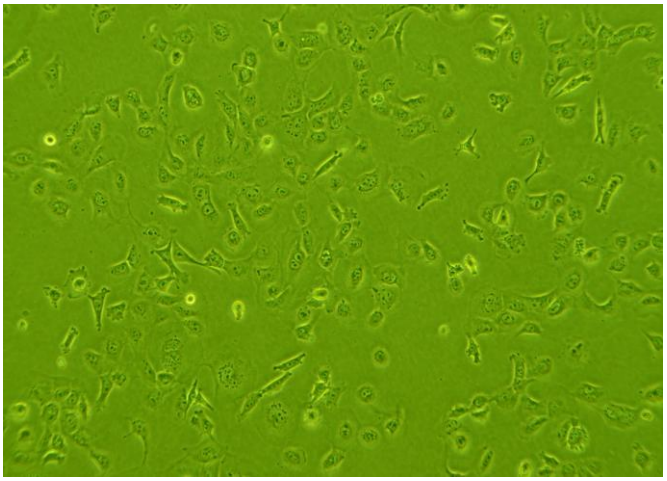
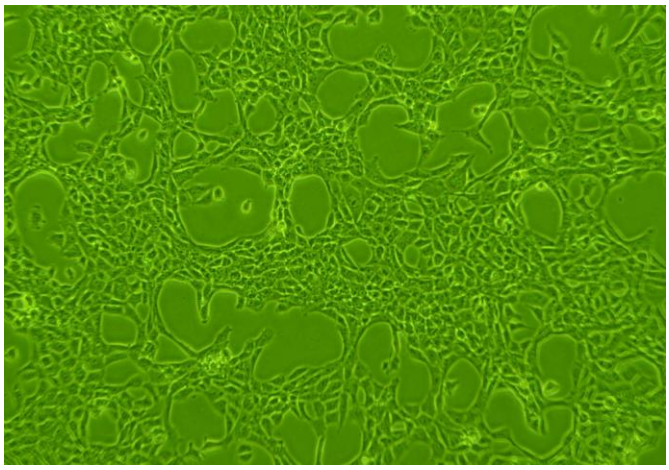
A 

B 

C 

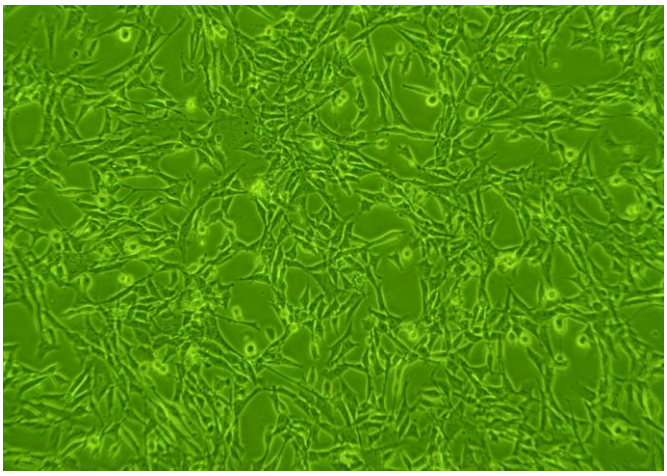
D 

Fig. 2.1. Morphology contrast of co-culture cells (A), Spermatogonia A cells (B), Leydig cells (C), and Sertoli cells (D). The co-culture cells were seeded in a 96-well plate at a density of 3.5×10^4 .Sertoli cells, Leydig cells and Spermatogonia A cells were seeded at a density of 2.0×10^4 cells per well, respectively, and then cultured for 24 h. The pictures were taken by digital camera under ten times (10x) magnification. Green filter was used. The single cell types were pure with no other kinds of cells. Spermatogonia A cells and Leydig cells appeared epithelial-like and polygonal with more regular dimensions. Sertoli cells were fibroblastic-like with multipolar and elongated shapes. The co-culture cells looks similar to Spermatogonia A cells due to its high percentage in the co-culture.

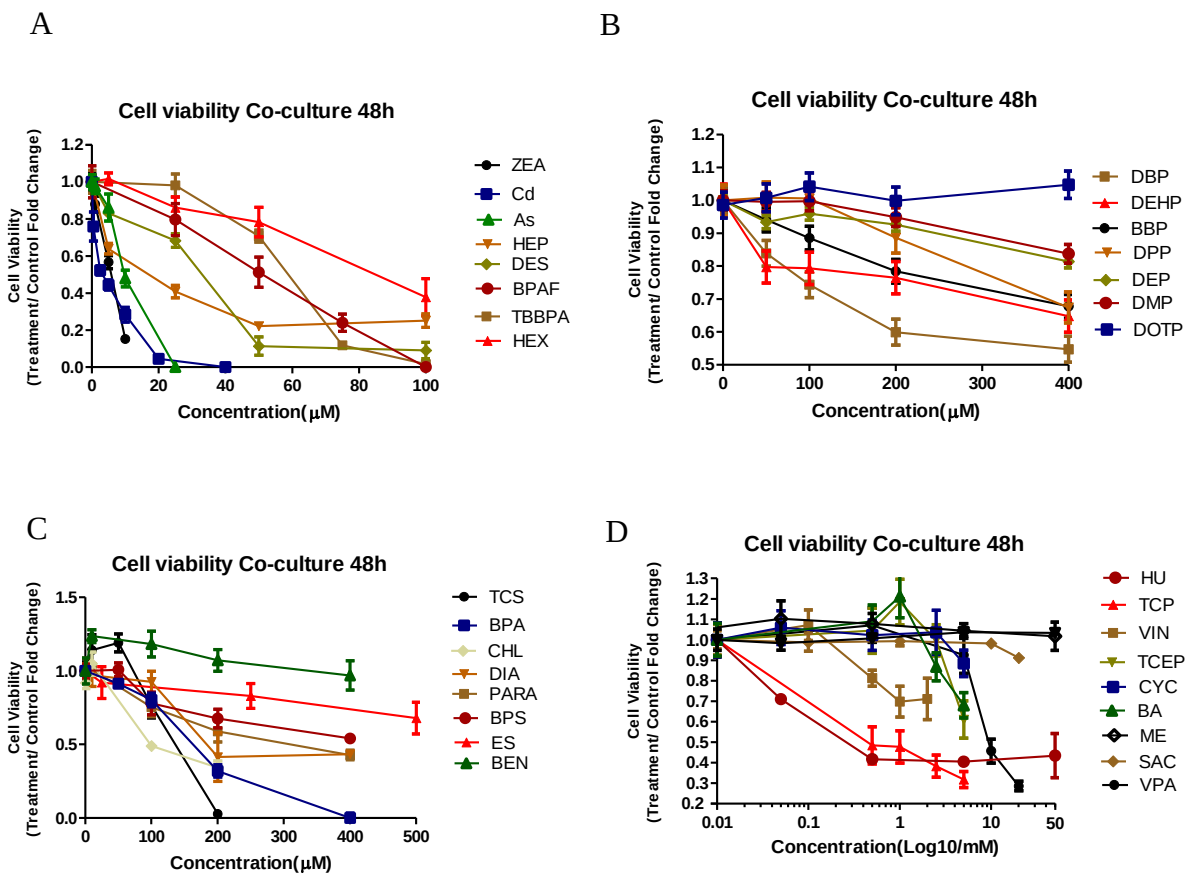


Fig. 2.2. Cell response to tested chemicals of co-culture at 48 h. The co-cultures were seeded in a 96-well plate for 24 h before treatment with chemicals at different concentrations for 48 h. Cell viability assessments were conducted using NR dye uptake assay. Data are presented as mean ± SD of five replicates. In order to show the dose response curves of these chemicals in an visually organized way, the chemicals were categorized into four groups based on the highest concentrations used; A at 100 µM, B at 400 µM, C at 500 µM, and D at 50 mM.

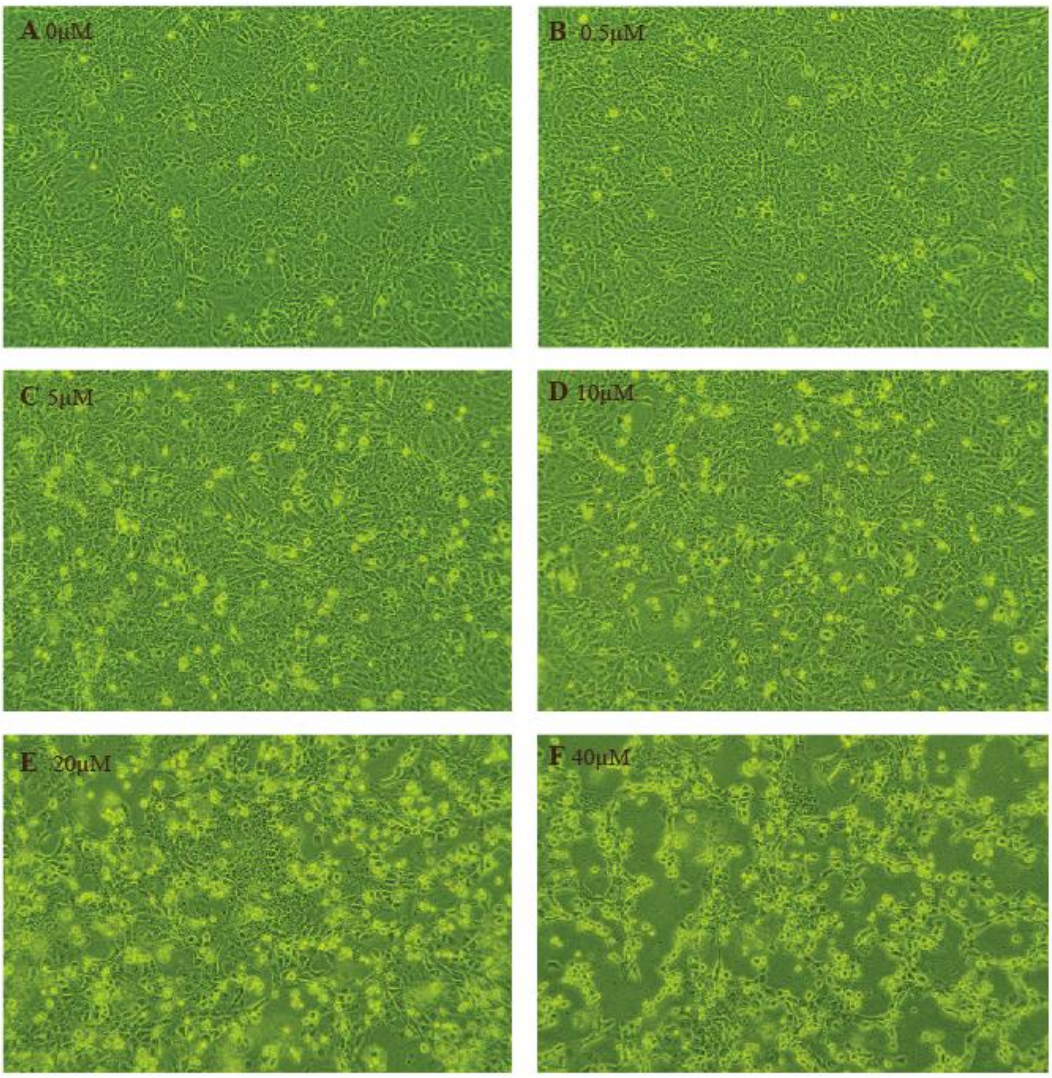


Fig. 2.3. Cd-induced dose-dependent morphological alternation of co-culture cells at 24 h. Co-culture cells were cultured in a 96-well plate overnight and then treated with Cd for both 24h and 48 h. The morphological changes of co-culture cells at 24 h treatment were shown. The panel refers to different concentrations of Cd: Control (A), 0.5 μM (B), 5 μM (C), 10 μM (D), 20 μM (E), and 40 μM (F). Green filter was used. The bright dots increased under treatment compared to control group. A dose-dependent disruption in cell morphology of co-culture cells was observed. The pictures were taken by digital camera under ten times (10x) magnification. Green filter was used.

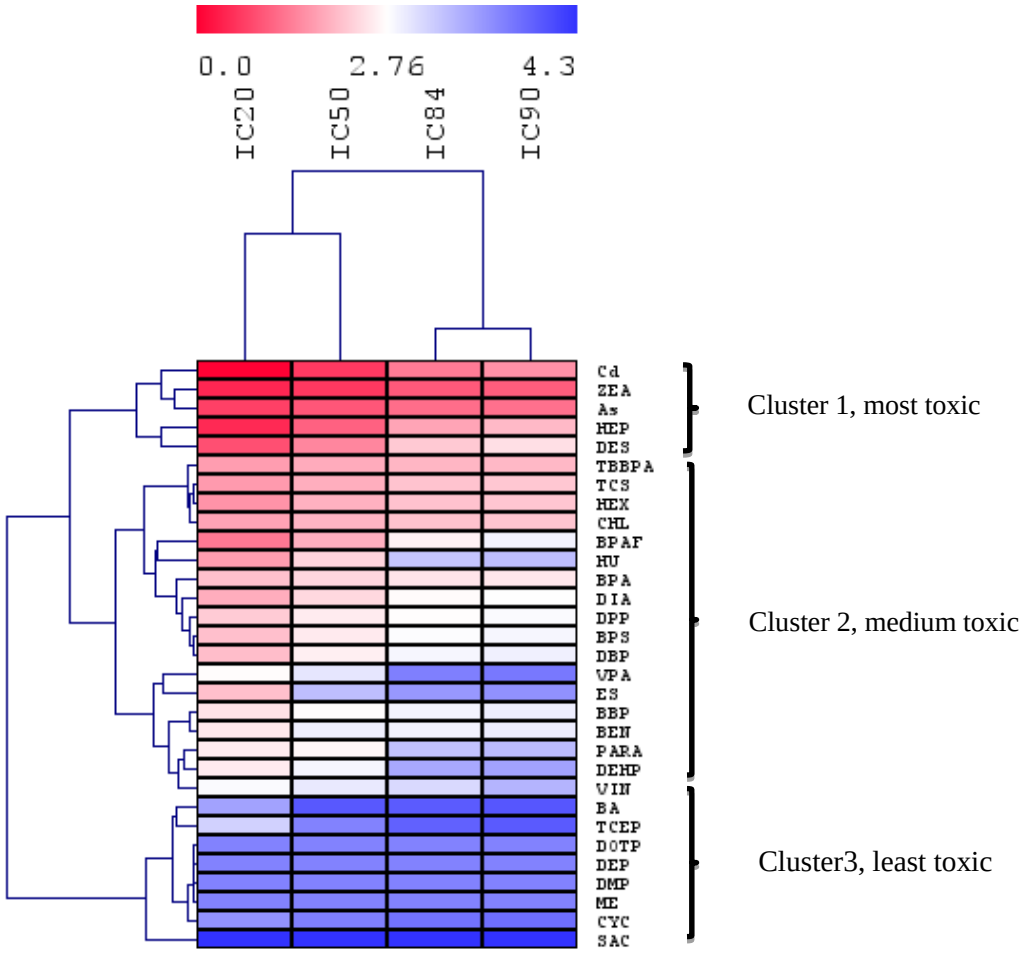


Fig. 2.4. Hierarchical cluster analysis for co-culture at 48 h. Non-supervised two-dimensional hierarchical clustering analysis of IC_{20}, IC_{50}, IC_{84}, and IC_{90} was conducted using the average linkage and elucidation dissimilarity method. IC_{20} to IC_{90} were log10-transformed before analysis. Gradient color indicates relative level of the log-transformed values. For chemicals of low toxicity, where IC values could not be derived through calculation, we used 5000 µM. The toxicity rankings of different categories of chemicals were assessed. For bisphenols: TBBPA > BPAF > BPA > BPS. For pesticides: As > HEP > TCS > HEX > CHL > PARA > VIN > BA. For phthalates: DPP > DBP > BBP > DEHP > DMP, DEP, DOTP.

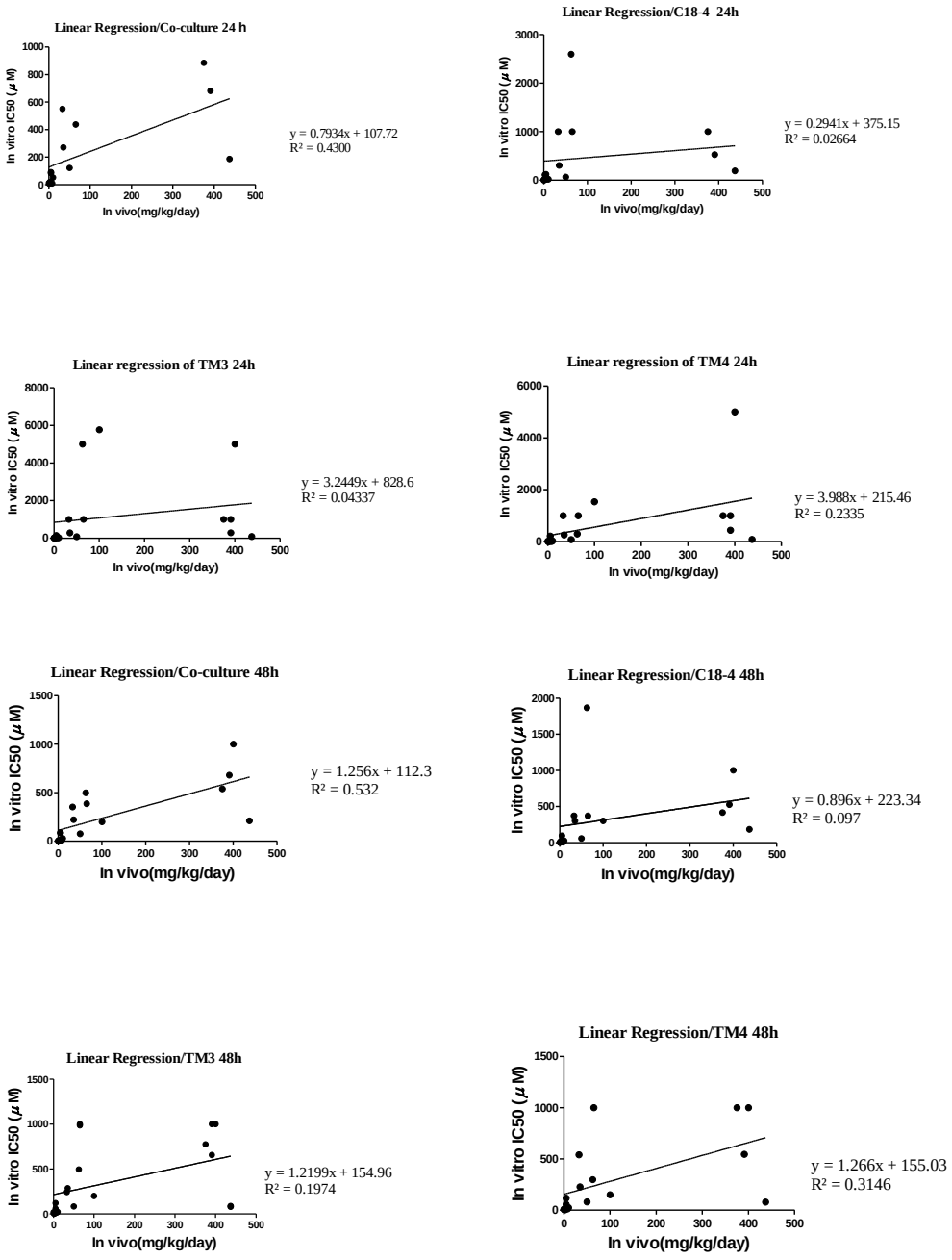


Fig. 2.5. Linear regression between rLOAEL values and IC_{50} values of co-culture and single cell types at 24 h and 48 h. Equation and R^2 are listed for each regression. Co-culture at 48 h had the highest R^2 value.

Table 2.1. List of chemicals tested. Chemical names, abbreviations, CAS numbers, molecular weight, purity, use of the compounds, and the source are summarized. There is no purity information for powdered chemicals (N/A).

Chemical Name	Abbreviation	CAS #	Molecular Weight	Purity (%)	Use	Provider
Sodium arsenite	As	7784-46-5	129.91	90	Pesticides	GFS Chemicals
Boric acid	BA	10043-35-3	61.83	99.5	Insecticide	Sigma
Benzyl butyl phthalate	BBP	85-68-7	312.36	98	Plasticizer	Sigma
Benzophenone-3,3′,4,4′-tetracarboxylic dianhydride	BEN	2421-28-5	322.23	96	Solvent	Sigma
2,2-Bis(4-hydroxy-3-methylphenyl)propane	BPA	80-05-7	256.34	99	Plasticizer	Sigma
4,4'-(hexafluoroisopropylidene)diphenol	BPAF	1478-61-1	336.23	97	Fire-retd plasticizer	Sigma
4,4′-Sulfonyldiphenol	BPS	80-09-1	250.27	98	Flame retardant	Sigma
Cadmium chloride	$CdCl_2$	10108-64-2	183.32	99.99	Dye	Sigma
Chlorpyrifos	CHL	2921-88-2	350.59	NA	Pesticides	ChemService
Cyclophosphamide	CYC	NA	279.1	NA	Cancer	TCI
Dibutyl phthalate	DBP	84-74-2	278.34	99	Solvent	Sigma
Dioctyl phthalate	DEHP	117-81-7	390.56	99.5	Plasticizer	Sigma
Diethylphthalate	DEP	84-66-2	222.24	99.35	Plasticizer	Sigma
Diethylstilbestrol	DES	56-53-1	268.35	99	Nonsteroidal estrogen	Fisher
Diazinon	DIA	333-41-5	304.35	NA	Pesticides	ChemService
Dimethyl phthalate	DMP	131-11-3	194.18	99	Plasticizer	Sigma
Dioctyl terephthalate	DOTP	6422-86-2	390.56	96	Plasticizer	Sigma
Dipentyl phthalate	DPP	131-18-0	306.4	99	Plasticizer	Sigma
β-Estradiol	ES	50-28-2	272.38	NA	Estrogen therapy	Sigma
Hexachlorophene	HEP	70-30-4	406.9	NA	Skin cleanser	Fisher
Heptachlor	HEX	76-44-8	373.32	NA	Pesticides	Fisher
Hydroxyurea	HU	127-07-1	76.05	98	Sickle cells	Sigma
2-methoxyethanol	ME	109-86-4	76.09	99.8	Solvent	Sigma

Parathion	PARA	56-38-2	291.26	NA	Pesticides	Chemservice
Saccharin sodium	SAC	128-44-9	205.17	NA	Sweetener	Sigma
3,3’,5,5’-tetrabromobisphenol A	TBBPA	79-94-7	543.87	97	Flame retardant.	Sigma
Tri-(2-chloroethyl)phosphate	TCEP	115-96-8	285.49	97	Fire-retd plasticizer	Sigma
Tricresyl phosphate	TCP	1330-78-5	368.36	90	Plasticizer	Sigma
2,4,4′-trichloro-2′-methoxydiphenyl ether	TCS	3380-34-5	303.57	NA	Bateria resistance	Sigma
Vinclozolin	VIN	50471-44-8	286.11	NA	Pesticides	Fisher
Valproic acid sodium salt	VPA	1069-66-5	166.19	98	Anticonvulsant	Sigma
Zearalenone	ZEA	17924-92-4	318.36	NA	Pesticides	Sigma

Table 2.2. IC_{50} values of tested chemicals in co-culture and single cell types at 24 h and 48 h.

Cell viabilities from NR dye uptake assay were calculated by dividing mean optical density (OD) values of treatment group by control group after subtracting the blanks (no cells). IC_{50}s were derived from dose-response curves with StatPlus using survival analysis and the probit method. For chemicals that the cell viability did not achieve 50% decrease at the highest treatment concentration, the highest concentration was assigned as IC_{50} (Nakatsu, Nakamura, Yamazaki, Sadahiro, Makuuchi, Kanno and Yamori, 2007).

	IC_{50} (μM)							
Chemical	Co-culture		Spermatogonia A		Leydig cells		Sertoli cells	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
ZEA	7.2	4.1	9.3	3.8	3.4	2.5	3.1	2.5
Cd	11.3	4.2	2.1	2.1	16.0	8.5	14.6	9.5
As	15.0	10.0	6.9	6.8	20.3	16.1	12.0	6.9
HEP	19.7	11.4	109.0	20.0	2.8	0.6	3.0	2.7
DES	51.1	29.0	25.5	18.0	32.4	23.0	25.9	24.1
TBBPA	70.0	58.0	74.9	55.0	70.0	60.0	60.0	38.0
TCS	121.0	76.5	62.7	57.3	135.0	80.0	81.0	72.7
BPAF	78.0	50.0	70.0	58.5	30.0	20.0	55.4	22.3
HEX	83.1	82.0	22.0	5.0	69.0	59.0	55.0	44.0
CHL	91.5	89.0	118.0	96.0	142.0	120.0	212.0	120.0
HU	1000.0	200.0	1000.0	300.0	600.0	200.0	396.0	150.0

BPA	210.0	186.0	190.0	184.5	88.0	80.5	89.0	79.0
DIA	270.0	222.0	303.0	200.0	286.0	250.0	256.0	227.0
BPS	574.0	341.0	505.0	357.0	435.0	400.0	211.0	74.3
DPP	550.0	352.5	400.0	372.0	400.0	244.0	400.0	400.0
DBP	437.0	387.5	400.0	371.0	400.0	400.0	400.0	400.0
PARA	469.0	400.0	343.0	307.2	400.0	400.0	294.0	117.5
TCP	5000.0	499.0	2929.0	1867.0	5000.0	600.0	298.0	163.0
BBP	885.0	538.9	400.0	400.0	400.0	400.0	400.0	400.0
DEHP	681.0	400.0	527.0	400.0	400.0	400.0	400.0	400.0
BEN	400.0	400.0	400.0	400.0	400.0	400.0	400.0	400.0
ES	1000.0	1000.0	1000.0	260.0	1000.0	927.0	1000.0	1000.0
VIN	4000.0	3600.0	1788.0	560.0	2000.0	2000.0	2000.0	2000.0
BA	6379.9	6071.0	5000.0	3582.0	19954.0	15623.0	5000.0	5000.0
TCEP	5000.0	5000.0	5111.0	3678.0	5000.0	5000.0	5000.0	5000.0
CYC	5000.0	5000.0	5000.0	5000.0	5000.0	5000.0	5000.0	5000.0
VPA	20000.	8850.0	20000.0	6000.0	20000.0	9481.0	20000.0	7606.0
SAC	20000.0	20000.0	20000.0	20000.0	20000.0	20000.0	20000.0	20000.0
ME	50000.0	50000.0	50000.0	50000.0	50000.0	50000.0	50000.0	50000.0
DEP	400.0	400.0	400.0	400.0	400.0	400.0	400.0	400.0
DMP	400.0	400.0	400.0	400.0	400.0	400.0	400.0	400.0
DOTP	400.0	400.0	400.0	400.0	400.0	400.0	400.0	400.0

Table 2.3. Benchmark dose (BMD) for each chemical in co-culture and single cell type at 48 h. 10% decrease of cell viability compared to controls was used to calculate BMD with EPA BMDS version 2.6. For chemicals with cell viability higher than 90% even at the highest concentration, the BMD cannot be calculated that the highest concentration tested was assigned to BMD.

Chemicals	BMD (μM)			
	Co-culture	Spermatogonia A	Leydig cells	Sertoli cells
ZEA	0.13	3.70	0.32	0.25
As	0.92	1.21	0.79	2.88
Cd	1.56	3.45	1.99	1.59
BPAF	1.71	1.07	1.07	1.41
DES	4.06	3.64	4.31	1.74
PARA	4.59	13.73	36.32	38.18
HEP	5.01	6.49	9.70	6.57
DBP	5.40	31.39	149.04	400.00
HEX	6.21	9.36	3.39	10.26
CHL	9.75	2.62	2.48	3.52
TBBPA	16.95	10.59	20.85	6.86
BPA	17.74	17.83	13.83	10.11
BPS	19.99	26.05	11.98	18.58
ES	29.13	62.87	53.34	53.13
DEHP	30.33	187.34	421.47	28.89
DIA	31.83	17.47	12.43	14.49
DPP	33.33	5.11	3.54	65.26
TCS	45.03	5.97	29.52	5.89
VIN	76.89	124.24	225.93	347.47
BBP	198.36	26.33	38.15	400.00
TCP	379.80	340.34	195.17	328.54
BEN	400.00	87.16	400.00	18.58
DEP	400.00	400.00	400.00	400.00
DMP	400.00	400.00	400.00	400.00
DOTP	400.00	400.00	400.00	400.00
VPA	1389.24	297.63	533.55	150.81
BA	2216.89	70.64	5000.00	423.78
HU	2278.89	2247.06	1914.89	1955.74
TCEP	3154.47	331.65	4554.02	3491.36

CYC	3290.05	2482.22	3500.00	3500.00
ME	5000.00	5000.00	5000.00	5000.00
SAC	20000.00	20000.00	20000.00	20000.00

Table 2.4. Pearson correlation between *in vivo* rLOAEL and *in vitro* IC_{50} in co-culture and single cell types. The toxicities of chemicals *in vivo* were indicated as rLOAEL (A). rLOAEL were derived from the ToxCast database. *In vivo* data were found for 18 chemicals. The Pearson correlation coefficient between rLOAEL and IC_{50} of co-culture and single cell types were calculated using GraphPad 5.0 (B). Pearson correlation coefficients are listed above. r = 0.2-0.4 denotes a weak association; r =0.4-0.6 denotes a moderate association; r = 0.6-0.8 denotes a strong association. Co-culture at 48 h gave the best correlation.

A

Chemical	*in vivo* (mg/kg/day)	24 h (μM)				48 h (μM)			
		Co-culture	Spermatogonia A	Leydig	Sertoli	Co-culture	Spermatogonia A	Leydig	Sertoli
ZEA	1	7.17	9.34	3.4	3.05	4.1	3.8	2.5	2.5
Cd	0.088	11.27	2.11	17.91	14.62	4.2	2.1	18	9.5
As	8	8.58	6.93	20.3	6.87	8.6	6.9	16.1	12
HEP	3	19.74	109.04	0.61	2.69	11.4	20	2.8	3
DES	10	51	18	32.35	26	29	25.5	23	24.1
TCS	50	121.01	62.68	82.99	77.29	76.5	57.3	83	81
HEX	5	83	18	69	41	83.1	18.1	59	55
CHL	5	91.54	118	142	212	89.1	96.3	120.3	117
HU	100	>1000	>1000	5771	1536	200	300	200	150
BPA	437	186	190	88	89	210	184.5	80.5	79
DIA	35	270	303	286	256	222	303	286.4	227
DPP	33	550	1000	1000	1000	352.5	372	244	540
DBP	65	437	1000	1000	1000	387.4	371	987	1000
TCP	63	>1000	2596	5000	298	499	1867	496	298
BBP	375	885	1000	1000	1000	538.9	417	775	1000
DEHP	391	681	527	1000	1000	681	527	657	545
VPA	400	>1000	>1000	5000	5000	1000	1000	1000	1000

B

Correlation	Co-culture	Spermatogonia A	Leydig cells	Sertoli cells
24h	0.6557 (p=0.0109)	0.1632 (p=0.5611)	0.2082 (p=0.4070)	0.4832 (p=0.0422)
48h	0.7296 (p=0.0009)	0.3114 (p=0.2237)	0.4443 (p=0.0497)	0.5609 (p=0.0192)

Table 2.5. Comparison of results from NR dye uptake assays and rLOAEL at 24 h and 48 h. For *in vitro* data, we defined $IC_{50} \leq 1{,}000$ µM as a positive result. For *in vivo* data, we defined rLOAEL ≤ 500 mg/kg/day as a positive result. Positive results are marked in red (+), and negative results in grey (-). NA = no available *in vivo* reproductive toxicity data in the ToxCast database. Concordance, sensitivity, and specificity between rLOAEL and IC_{50} of co-culture and single cell types were calculated (B). The 95% confidence intervals are also listed. Concordance is the percentage of NR dye uptake results that matches *in vivo* data (both positive and negative). Sensitivity is the proportion of chemicals with positive IC_{50} *in vitro* where the corresponding rLOAEL is also positive *in vivo*. Specificity is the proportion of chemicals with negative IC_{50} *in vitro* where the corresponding rLOAEL is also negative *in vivo*. Co-culture at 48 h showed the highest concordance, sensitivity, and specificity.

A

Chemical	*In vivo* (mg/kg/day)	IC_{50}(µM)							
		24 h				48 h			
		Co-culture	Spermatogonia A	Leydig cells	Sertoli cells	Co-culture	Spermatogonia A	Leydig cells	Sertoli cells
DIA	+	+	+	+	+	+	+	+	+
DES	+	+	+	+	+	+	+	+	+
Cd	+	+	+	+	+	+	+	+	+
CHL	+	+	+	+	+	+	+	+	+
HU	+	+	+	+	+	+	+	+	+
HEP	+	+	+	+	+	+	+	+	+
HEX	+	+	+	+	+	+	+	+	+
As	+	+	+	+	+	+	+	+	+
ZEA	+	+	+	+	+	+	+	+	+
TBBPA	+	+	+	+	+	+	+	+	+

BPAF	+	+	+	+	+	+	+	+	+
BPA	+	+	+	+	+	+	+	-	+
TCS	+	+	+	+	+	+	+	-	-
DEHP	+	+	-	-	-	+	+	+	+
DBP	+	+	-	-	-	+	+	+	-
TCP	+	-	-	-	+	+	-	+	+
BBP	+	+	-	-	-	+	+	+	-
DPP	+	+	-	-	-	+	+	+	+
VPA	+	-	-	-	-	+	-	-	-
TCEP	+	-	-	-	-	-	-	+	+
PARA	+	-	+	-	+	+	+	+	+
Me	+	-	-	-	-	-	-	-	-
VIN	+	-	-	-	-	-	-	-	-
BA	-	-	-	-	-	-	-	-	-
DOTP	-	-	-	-	-	-	-	-	-
DMP	-	-	-	-	-	-	-	-	-
DEP	-	-	-	-	-	-	-	-	-
SAC	-	-	-	-	-	-	-	-	-
ES	NA	-	-	-	-	-	+	+	-
CYC	NA	-	-	-	-	-	-	-	-
BPS	NA	+	+	+	+	+	+	+	+
BEN	NA	-	-	-	-	+	-	-	-

B

	24 h				48 h			
	Co-culture	Spermatogonia A	Leydig cells	Sertoli cells	Co-culture	Spermatogonia A	Leydig cells	Sertoli cells
Concordance	65.63% (45.72%, 79.27%)	48.57% (32.01%, 65.13%)	44.44% (28.21%, 60.68%)	52.94% (36.16%, 69.72%)	79.31% (64.57%, 94.05%)	73.33% (57.51%, 89.16%)	73.33% (57.51%, 89.16%)	67.74% (51.29%, 84.20%)
Sensitivity	71.43% (52.11%, 90.75%)	57.14% (35.98%, 78.30%)	52.38% (31.02%, 73.74%)	69.72% (41.13%, 82.68%)	85.71% (70.75%, 100%)	80.95% (64.16%, 97.75%)	80.95% (64.16%, 97.75%)	76.19% (57.97%, 94.41%)
Specificity	45.46% (16.03%, 74.88%)	35.71% (10.61%, 60.81%)	33.33% (9.48%, 57.19%)	38% (12.02%, 64.91%)	62.50% (28.95%, 96.05%)	55.56% (23.09%, 88.02%)	55.56% (23.09%, 88.02%)	50% (19.01%, 80.99%)

CHAPTER 3

CONCLUSIONS

In this study, we proposed to construct a co-culture system which was developed previously in our group by using cell lines instead of primary cells to reduce the usage of animals. We have chosen spermatogonia A cells, Leydig cells and Sertoli cells to construct this co-culture system. The results suggest that this model may serve as an alternative to recognize chemicals' reproductive toxicity in vitro.

We confirmed that a co-culture was better than single cell types in mimicking testes function after comparing our data with *in vivo* rLOAEL values. The co-culture includes three sensitive testes cell types, allowing communication between them. Due to the complexity of testes, a co-culture should be better at mimicking testes function than a single cell type. Our results demonstrate that co-culture cells correlate better with *in vivo* rLOAEL values than single cell types. The co-culture displayed the highest R^2 value in linear regression analysis and the highest *r* value in Pearson's correlation coefficient analysis. The results support our hypothesis.

The co-culture distinguished between chemical toxicities in different categories, and, more importantly, allowed the ranking of chemical toxicity by cluster analysis. Three clusters were identified, defined as very toxic, medium toxic, and least toxic. We found that the results were consistent with published *in vivo* data. rLOAEL values of Cd, ZEA, As, DES, and HEP were 0.88. 1, 8, 10, and 3 mg/kg/day, respectively, indicating their high toxicity to animals; they were defined as very toxic. DEP, DOTP, and DMP, which ranked as least toxic, are known as developmentally non-toxic phthalates.

For testing new chemicals, cluster analysis can assist in predicting their toxicity levels. The toxicity level of different categories of chemicals was also found. For bisphenols, the toxicity ranking was: TBBPA > BPAF > BPA > BPS. For pesticides, the ranking was: As > HEP > TCS > HEX > CHL > PARA > VIN > BA. For phthalates, the toxicity ranking was: DPP > DBP > BBP > DEHP > DMP, DEP, DOTP.

Also, the chemicals' toxicity was time-dependent. Chemicals were generally more toxic at 48 h than 24 h for both co-culture and single cell types. This is also true for the *r* value of the Pearson correlation coefficient and the R^2 value of linear regression. We conclude that treatment time greatly influences the toxicity of chemicals. In future studies, ore time point should be tested.

Different cell types display different sensitivities toward a given chemical, as indicated by the IC_{50}. Leydig cells were more sensitive to DPP in single cell types, suggesting that DPP probably be an endocrine disruptor (Kumar, Srivastava and Roy, 2015). The IC_{50} values of BPA, TCP and Cd to Sertoli cells were lower than for other cell types, indicating that their target may be Sertoli cells. Results from treating single cell types may provide some hint on chemicals' target *in vivo*.

In summary, by using cell lines to construct a co-culture model, we demonstrated the possibility of distinguishing testicular toxic from non-toxic chemicals. Moreover, highest correlation with *in vivo* rLOAEL was found in the co-culture model at 48h treatment. The calculation of concordance, sensitivity, and specificity further supported the reliability of this model. We have demonstrated that our *in vitro* co-culture model may be useful in screening testicular toxicants in a wide concentration range and prioritize chemicals for future experiments.