THE UTILIZATION OF EMBRYONIC STEM CELLS TO DISCERN EMBRYOTOXICITY *IN VITRO*

By

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Abstract

The terms developmental toxicity and embryotoxicity are becoming prominent in today's research, drawing attention to the harmful toxicants pregnant women are exposed to on a day-to-day basis. Chemicals in industry and in the environment, as well as consumer products can critically hinder tissue maturation and metabolism of the fetus. Currently, adverse effects of chemical exposure are tested using *in vivo* models. However, to spare animals, *in vitro* methods have been developed, among them the Embryonic Stem Cell Test (EST) that uses embryonic stem cells (ESCs). ESCs are extracted from the inner cell mass of a blastocyst and can model various stages of embryogenesis, while replicating the conditions and cell-cell signals eminent in early development. The EST uses three toxicological endpoints to compare the extent of a chemical's influence on development: cytotoxicity in ESCs, differentiation inhibition in ESCs, which both act as a surrogate for embryo development, and cytotoxicity on fibroblasts, which represent maternal effects. Concentration-response curves and linear discriminant analysis are then able to identify the potency of a chemical to cause developmental problems, ranging from insignificant effects to posing threats to vital functions during the fetal stage. Originally utilized with mouse cells, the predictivity of this model for human risk assessment remains questionable. Therefore, this work will identify the differential predictivity of human cells by testing a small battery of chemicals in both model systems.

Acknowledgements

I would like to acknowledge my faculty mentor Dr. Nicole zur Nieden for all her support throughout my University Honors capstone project. Without her guidance and encouragement I would not have had the opportunity to explore research and publish this review. I would also like to acknowledge my graduate student mentor, Madeline Vera-Colon, for her unwavering support and patience in the lab. Additionally, I would like to thank the University Honors Program for providing me with a platform to present my scientific explorations.

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Nature and Purpose

Embryotoxicity is a prominent field of scientific research which draws attention to the toxicological changes in our day-to-day lives, and how this can directly influence development of the embryo during pregnancy. Chemicals found in the industry, environment, and within consumer products can critically hinder tissue maturation and metabolism of the fetus. Exposure to these harmful chemicals can directly cause problems within the embryo. Specifically, they can cause skeletal abnormalities and, in many cases, cause embryo-fetal deaths.

However, stressors to maternal tissue are also proven to cause indirect consequences to early embryonic gestation. Around 20% to 40% of infant deaths are caused by developmental issues involving interference to the maternal uterus (Buck et al., 2018). For example, various vaginal carcinomas may affect the critical periods of fetal development. Exposure of the expectant mother to harmful chemicals may cause maternal calcium deficiencies that harm fetal bone formation and maturation through placental transfer. These stressors, in some cases, can even have adverse effects on the maturation of cells into adulthood of the offspring. Environmental chemical exposure can also disrupt the natural production of hormone cycles and can still cause damage after gestation to the infants through breastfeeding (Hong et al., 2010).

Thus, by assessing the detrimental impacts of novel chemicals in our environment we are able to take a deeper look into our changing industrial landscape and its impact on future generations. Synthetic agents are becoming more predominant in the industry, and many big companies are neglecting the effects of these chemicals. Heavy metals and chemicals, such as iron and quercetin, are potent toxicological agents causing a wide range of developmental malformations. Their omnipresent exposure in the environment is a vital topic of research, especially when observing the densities of these chemicals in relation to their effects on fetal

development. In many cases, even small concentrations of toxic compounds can penetrate the estrogenic barrier shared between the mother and the fetus (Alder et al., 2008). For example, monomers, such as those in plastics, resins and beverage containers, contain a multitude of agents that may potentially be harmful to the pregnancy (Hong et al., 2010). New methods of consumer creation in the industry is a beneficial way to observe economical growth, but to what degree is it affecting the prosperity of the future generations?

To answer this question, we can use a relevant *in vitro* tool to assess the outcome of chemical exposure on skeletal development with greater predictability to human health through human ESCs. This allows us to create standard research models for various health detriments caused by our environment. Implementing various experimental tests to determine the teratogenic effects of environmental toxicological agents, we are able to explore osteogenesis *in vitro* by recapulating embryonic development.

Background

Currently, adverse effects of chemical exposure are typically tested using *in vivo* models. However, to spare animals, *in vitro* methods have been developed, among them is the Embryonic Stem Cell Test (EST) that uses embryonic stem cells (ESCs) (Heuer et al., 1999). ESCs are extracted from the inner cell mass of a blastocyst and can model various stages of embryogenesis, while replicating the conditions and cell-cell signals eminent in early development. The EST uses three toxicological endpoints to compare the extent of a chemical's influence on development: cytotoxicity in ESCs, differentiation inhibition in ESCs, which both act as a surrogate for embryo development, and cytotoxicity on fibroblasts, which represent maternal effects (Seiler et al., 2011; Kleinstreuer et al., 2011). Concentration-response curves and linear discriminant analysis are then able to identify the potency of a chemical to cause

developmental problems, ranging from insignificant effects to posing threats to vital functions during the fetal stage. Originally developed with mouse ESCs, the predictivity of this model for human risk assessment is questionable. Nonetheless, the gradual popularity of the use of ESCs can reduce the cost-intensive aspect associated with an *in vivo* model. *In vitro* evaluation will help us relatively accurately model the earliest stages of development, while observing many critical timepoints during post-implantation chemical exposure (Madrid et al., 2018). Compounds that are highly embryotoxic can be identified with the EST at high accuracy (Scholz et al., 1999a and 1999b) and would not need to be tested in an *in vivo* study.

The ease of extraction, developmental potential, and utility of ESCs make them an ideal candidate to assess the risks associated with toxicological chemicals a pregnant mother might encounter on a daily basis. Under specific conditions, ESCs have the ability to become highly specialized (Itzkovitz-Eldor et al., 2000), allowing researchers to examine the effects of various compounds on the development of these cells. Any triggers that disrupt the full specialization of human ESCs can be attributed to the experimental changes done to inhibit the possible differentiation (Ko et al., 2019). Since we are specifically looking at the chances of bone deformities due to the exposure of a certain harmful chemical, we can observe osteogenic capabilities in ESCs. This is possible because differentiation protocols have been developed for both mouse and human ESCs. These generate mature osteoblasts capable of mineralizing the extracellular matrix (zur Nieden et al., 2003; Sparks et al., 2018), a hallmark feature of bone cells. Observing this calcium formation in the osteogenic ESCs may then serve as an endpoint to identify the embryotoxic potential of the chemical during toxicological screenings (zur Nieden et al., 2004; zur Nieden et al., 2010; Madrid et al., 2018). Since both the mouse and human cells

exhibit the matrix mineralization when osteogenically differentiated, the aim of this study is to test whether the mouse or human cells can more accurately determine human exposure risk.

Previous Results

To establish a proper baseline for this study, the lab has previously employed reference chemicals - chemicals with known effects on embryonic development - and tested them on both mouse and human cells. Penicillin G and isoniazid acted as negative chemicals (non-embryotoxic), whereas 5-fluorouracil (5FU) and all-trans retinoic acid (atRA) are strongly embryotoxic chemicals (Zurlinden et al., 2020). To determine whether the reference chemicals elicited the expected responses, the lab tested all four chemicals in a wide range of concentrations using all three EST endpoints on both cell types. Cytotoxicity was evaluated with an MTT assay and differentiation inhibition assessed with a calcium assay. Obtained values were expressed as percentage of the solvent-exposed cells. Half-maximal inhibitory doses were then established from those concentration-response curves and input into a biostatistical classification model (Genschow et al., 2000). This biostatistical model was developed with a set of 20 chemicals with known embryotoxicity potential and can thus classify unknown chemicals into three distinct embryotoxicity classes - non-embryotoxic, weakly embryotoxic and strongly embryotoxic. In these reference chemical experiments, all four chemicals were classified according to their known embryotoxicity potential. However, there was a greater sensitivity to the chemical tested when analyzing the IC_{50} and ID_{50} dose-response curves in the human ESCs when compared to the mouse ESCs. Together, this previous work established the utility of the selected in vitro systems and already gave the first hint that the sensitivity of the human cells might be higher than that of the mouse cells.

Methods

Cell culture Preparations

Mouse D3 ESCs (American Type Culture Collection (ATCC), Rockville, MD, USA) were routinely grown in the presence of Leukemia Inhibitory Factor (LIF, 1000U/ml, Gibco) at 37 °C with 5% CO_2 to maintain their undifferentiated status and passaged every second day. ESC medium consisted of high glucose DMEM (4.5 g glucose/l, Gibco) supplemented with 20% fetal bovine serum (FBS, Sigma, chosen batches), 50 U/ml Penicillin and 50 µg/ml Streptomycin, 1% non essential amino acids (Gibco) and 0.1 mM -Mercaptoethanol (Sigma).

H9 human embryonic stem cells (hESCs) were cultured on Matrigel (BD Biosciences) treated culture plates in mTeSR 1 medium (Stem Cell Technologies) at 37 °C with 5% CO_2. Colonies were passaged every 3 days using accutase (2–4 min at RT) and a cell scraper to displace colonies from the plastic well. This allows the process of proper passaging as it maximizes the transfer of viable cells into the new well and maintains the hESCs in an undifferentiated stem cell state.

Mouse 3T3 fibroblasts (ATCC) and human foreskin fibroblasts (hFF, kind gift of Dr. Derrick Rancourt, University of Calgary, Canada) were maintained in DMEM with 10% FBS, 1% (v/v) non-essential amino acids, 50 U/mL penicillin and 50 µg/mL streptomycin) and plated into a 0.1% gelatin-coated plate. Cells were passaged every 3 days using 0.25% Trypsin-EDTA (5 min at 37C).

Osteogenic differentiation of embryonic stem cells

Mouse ESCs were osteogenically induced as described (zur Nieden et al., 2003) through the formation of embryoid bodies in control differentiation medium (CDM). CDM was composed of DMEM, 15% FBS (Atlanta Biologicals), 1% (v/v) non-essential amino acids, 50 U/mL

penicillin, 50 µg/mL streptomycin, and 0.1 mM β-mercaptoethanol. On day 5 of the differentiation protocol, embryoid bodies were dispersed with Trypsin/EDTA and seeded into osteogenic differentiation medium (CDM containing $5*10^{-8}$ M 1,25α$(OH)_2$ Vitamin D_3 (VD_3; Calbiochem), 0.1 mM β-glycerophosphate, and 50 µg/mL ascorbic acid) (zur Nieden et al., 2003).

To induce osteogenic differentiation in H9 hESCs, these cells were grown to confluence of about 70% at which point CDM was added (designated d0). After the first five days of differentiation, CDM was supplemented with $1.2*10^{-7}$ M 1,25α$(OH)_2$ Vitamin D3 (VD3; Calbiochem), 0.1 mM β-glycerophosphate, and 20.8 µg/mL ascorbic acid (Sparks et al., 2018). These additional supplemental nutrients force the cells to adopt an osteogenic lineage fate.

Selection of test chemicals

Folic Acid, Coumarin, 17α-Estradiol, and Acetaminophen were chosen as test chemicals for this study. Folic acid, which is a main component in newborn prenatals, is seen to cause some birth defects under constant exposure (Ostrea et al., 2022). An active ingredient in cinnamon, Coumarin can cause damaging birth defects such as chondrodysplasia during fetal development (Wang et al., 2013). 17α-Estradiol is a topical medication which is used to treat hair loss. This is also a well known ingredient in oral contraceptives which can cause problems in conception and pregnancy if not taken according to the dosage regulated (Thomas et al., 1983). Acetaminophen, on the other hand, is a very common pain reliever found in drug stores and can be easily requested over-the-counter. Tylenol, the generic name for is an example of acetaminophen, which can be used to treat minor fevers to body aches (Bauer et al., 2021). Over the years, much debate surrounds the question as to whether acetaminophen is safe for consumption during pregnancy and whether it has direct consequences to fetal development. Recent studies by the

EPA show that the effects of acetaminophen are under close study but nonetheless are actually shown to increase the risk of neurodevelopmental issues with the unborn child. All of the chemicals chosen for ESC experimentation seem to come from different pharmacological backgrounds. However, it is important to note that all of these chemicals are under strict EPA and FDA guidelines as having the intrinsic capability to cause intrinsic harm to the development of the fetus. Reproductive and urogenital diseases were commonly associated in some cases of women who were in close contact or who have ingested these chemicals in an exceeding amount.

Chemical exposure

Test chemicals were delivered throughout the entire duration of differentiation with the appropriate concentrations prepared beforehand. All test chemicals were purchased from Sigma-Aldrich and dissolved in DMSO at a stock concentration of 100,000 µg/mL. Working stocks were diluted down to 10,000 µg/mL in DMEM. All of the stocks were stored in -20 °C. Final dilutions were made in appropriate cell culture medium to the tested concentrations. This action prevents cross contaminations of the concentration studied, providing a more accurate result of the effect of the chemical. Exposure started on day 0 of the differentiation and continued through day 20 in all cells tested. Medium, including chemical dilutions, were made fresh and changed every other day. This process ensured that the cells did not die and were under constant exposure to the chemical at hand. Very controlled sterilization methods were in place to prevent the ESCs coming into contact with bacteria, as this would impact the differentiating capabilities of the cells.

Detection of calcium

Quantification of calcium content was performed using a calcium assay normalized to protein content (Davis et al., 2011). Cells were lysed with radio-immunoprecipitation (RIPA) buffer (1% NP40, 0.5% sodium deocycholate, 0.1% sodium dodecyl sulfate in phosphate-buffered saline (PBS)) on day 20 of cellular differentiation. Remaining matrix was washed with 1 N HCl and collected. Both lysates were assayed with Arsenazo III (Genzyme), and absorbance was measured at 655 nm (iMark microplate reader; BioRad). Absorbances were compared to a $CaCl_2$ standard and total calcium content was normalized to total protein content determined by a Lowry assay. Lowry assay was read at 750 nm (iMark microplate reader; BioRad) after a 15-minute micro-shake incubation and total protein was determined by comparing to a bovine serum albumin (BSA) standard curve.

MTT assay

Viability response to the chemical exposure was determined through a 3-[4,5-dimethylthiazol-2yl]-2,5-diphenylterazolium bromide (MTT) assay which was used the generate the IC_{50} curve at time of data analysis.. Cells were incubated with MTT solution (5 mg/ml) at 37 °C for 2 h. Following incubation, the supernatant was removed and replaced with 0.04 mol/L HCl in isopropanol. The plate was placed on a shaker for 15-minutes to dissolve aggregates. The optical density of the solution was read at 595 nm (iMark microplate reader; BioRad) (zur Nieden et al., 2010; zur Nieden & Baumgartner, 2010; Walker et al., 2014). Reading the MTT assay allows us to gather the state of differentiation in the cells while measuring their viability.

Statistical analysis

Half-maximal inhibitory doses of cytotoxicity (IC_{50}) and osteogenic differentiation (ID_{50}) were taken from concentration-response curves (nonlinear regression; GraphPad Prism) and embryotoxicity classes calculated according to (Genschow et al., 2002). A biostatistical prediction model based on linear discriminant functions was employed to correctly classify the test chemical into one out of three total embryotoxicity categories (strong, weak, and non-embryotoxic) (Genschow et al., 2002). In this model, calculated ID_{50} concentrations are relatively compared to the chemical IC_{50} counterpart. All exposures were performed in biological quintuplicate.

Experimental design

The practicality of using ESCs outweigh the related ethical concerns due to the drastically reduced direct animal intervention (Gallego et al., 2014). The similarities between ESCs and the conditions emulated to test toxicology open new pathways of research, helping develop stem cells into mature cells. This unfolds a wide range of differentiating capacity human ESCs hold, which can be accommodated to test a plethora of screening experiments (Borras-Granic et al., 2014).

This study includes multiple endpoints of assessment of the effect that a test chemical elicits on cellular growth and differentiation. When taking a deeper look into the experimental analysis, we can see that the cytotoxicity of the chemical is assessed on osteogenically differentiating ESCs. Through this, we focus on growth inhibition, survival defects and possible cell death. This is contrasted with a calcium assay that evaluates the efficiency of cellular differentiation. Through this assay, we focus on the possible differentiation inhibition caused by

chemical exposure. Thirdly, cytotoxicity is tested in a fibroblast cell line to assess viability issues caused by the chemicals.

Initial stages of ESC development, which are identified as days 0-4, are used as markers to indicate the decreased pluripotent ability in these cells. During the progression of the experiment, the H9 cell line is a presumed model of the human embryo, in which the extent of toxicity to bone development is studied. In conjuncture, the fibroblasts will be a model of fully differentiated cells, which mimic the effects on the fully developed cells in the mother (Walker et al., 2014). By observing both the H9 and HFF cell lines, we are able to gauge the effects of the chemical tested on the development of the fetus, while noting the direct impacts the chemical might have on the full differentiated mother. Through this, we are able to test the malleability of the embryonic barrier between the mother and the fetus.

Half-maximal inhibitory concentrations taken from the resulting concentration-response curves will be used to assign an embryotoxicity class to the test chemical. In addition, the half-maximal inhibitory concentrations will further help uncover the differential sensitivity of the human ESCs versus the mouse ESCs.

Over the period of twenty days, which is the determined time for full ESC development before they become completely specialized osteoblasts (Sparks et al., 2018), they must undergo media changes every other day (Tandon et al., 2012). As detailed in the experimental design, the media contained vital nutrients under the exposure of decreasing concentrations, by a ten fold, in addition to the untreated control media. Ensuring the suspension of proper media will help the cells grow in their respective chemical exposure concentrations, as labeled from the lowest concentration of 0.0001 to the highest concentration of 1000 μg/mL. Testing the chemical of

interest in these concentrations will ensure accurate predictability to the effects on fetal development in case of a toxicological exposure.

By having five wells dedicated to each of the media concentrations being tested, we were able to gather the needed data from the cells while accounting for experimental error and ensuring statistical rigor. We would also make sure to observe the cells under the microscope during these media changes to ensure proper growth of the differentiated cells. While differentiation experiments were ongoing, it was vital to passage the undifferentiated cells to ensure the growth of new maintenance wells so they are available for the next chemical exposure (Boras-Granic et al., 2014).

Results

Folic Acid, Coumarin, 17α-Estradiol, and Acetaminophen were the chemicals selected for testing. Folic Acid exposure was at chemical concentrations ranging from 0.00001 µg/mL to the highest concentration of 1000 µg/mL in both mouse and human *in vitro* models. The MTT assay yielded a resulting IC_{50}, which determined the half-maximal concentration at which 50% of the cells were non viable. In turn, the resulting ID_{50} reflected the chemical concentration at which 50% of the cells experienced a decrease in their osteogenic differentiation capacity. This was indirectly quantified by measuring calcium content via a calcium assay normalized to protein content. In the mouse *in vitro* model, the IC_{50} concentration for folic acid was determined to be 194.8 µg/mL within the fibroblasts (Figure 1). However, no IC_{50} or ID_{50} could be established for the osteogenically differentiating mESCs in the tested concentration range. In turn, within the human *in vitro* model, the ID_{50} concentration was calculated at 5.850 µg/mL while the H9 MTT IC_{50} was greater than 1,000 µg/mL and the hFF MTT IC_{50} was at 5.076 µg/mL (Figure 2).

In Coumarin exposure, concentrations ranged from 0.00001 µg/mL to the highest concentration of 10 µg/mL. In the mouse model, the IC_{50} concentration was determined at 6.542 µg/mL for the mESCs MTT while the 3T3 MTT had an IC_{50} of 0.2040 µg/mL (Figure 3). An ID_{50} could not be established within the tested concentration range. In the human cell line, the ID_{50} concentration was found at 0.01531 µg/mL while the H9 MTT IC_{50} was at 0.01518 µg/mL and the hFF MTT IC_{50} was at 0.05810 µg/mL (Figure 4).

In the 17α-Estradiol exposure, similarly, concentrations exposed ranged from 0.00001 µg/mL to the highest concentration of 1000 µg/mL. In the mouse model, the IC_{50} value was calculated to be 8.445 µg/mL for the 3T3 MTT (Figure 5). Again, half-maximal inhibitory doses for the mESCs could not be determined within the tested concentration range. However, within the same tested concentration range, all half-maximal inhibitory doses were found for the human cells. The ID_{50} value for the H9 calcium content was 4.470 µg/mL while the H9 MTT IC_{50} was at 10.13 µg/mL. The hFF MTT for the IC_{50} was at 10.64 µg/mL (Figure 6).

In the mouse model of Acetaminophen-exposed cells, the IC_{50} concentration was calculated to be 6.621 µg/mL for the D3.6 MTT while the 3T3 MTT IC_{50} was at 5.725 µg/mL (Figure 7). THe ID_{50} for the mESCs could not be established. In the human cell lines, the ID_{50} concentration for the H9 Calcium/Protein test was at 6.364 µg/mL while the H9 MTT IC_{50} was at 9.986 µg/mL. The hFF MTT IC_{50} was at 15.33 µg/mL (Figure 8).

Figures

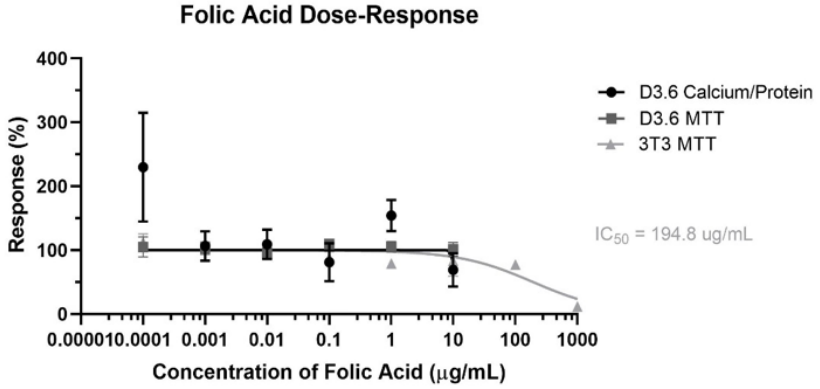

Figure 1: Mouse Embryonic Stem Cell Model - Folic Acid graphs were plotted and ID_{50}/IC_{50} values were calculated using non-linear regression analysis via GraphPad Prism.

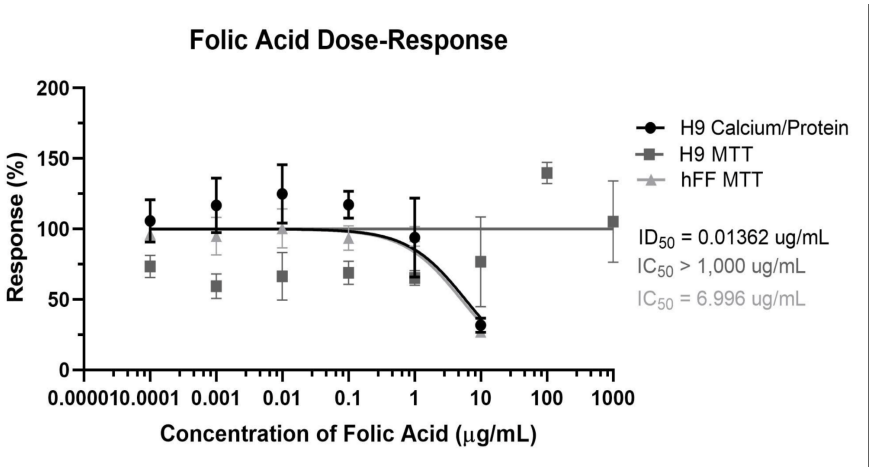

Figure 2: Human Embryonic Stem Cell Model - Folate graphs were plotted and ID_{50}/IC_{50} values were calculated using non-linear regression analysis via GraphPad Prism.

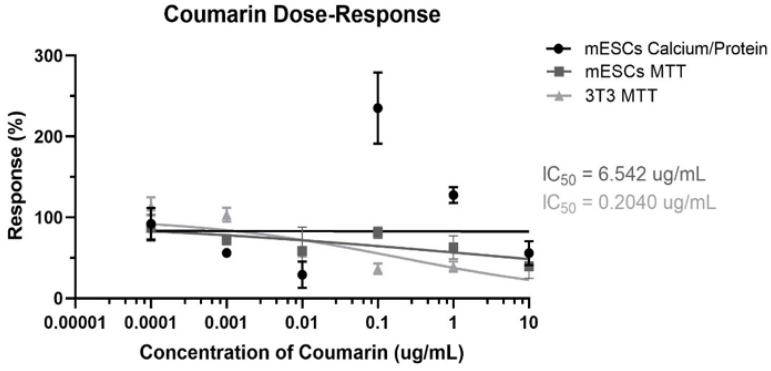

Figure 3: Mouse Embryonic Stem Cell Model - Coumarin graphs were plotted and ID_{50}/IC_{50} values were calculated using non-linear regression analysis via GraphPad Prism.

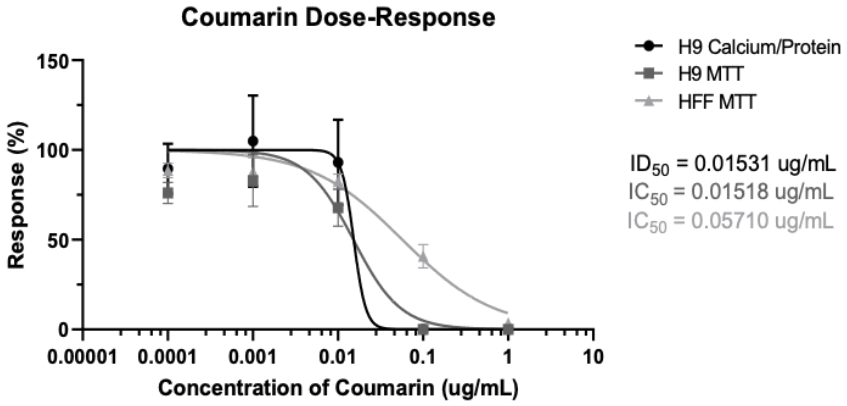

Figure 4: Human Embryonic Stem Cell Model - Coumarin graphs were plotted and ID_{50}/IC_{50} values were calculated using non-linear regression analysis via GraphPad Prism.

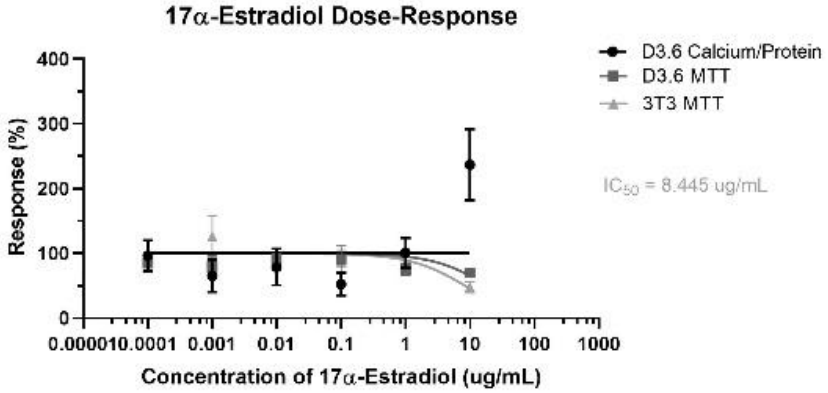

Figure 5: Mouse Embryonic Stem Cell Model - 17a-Estradiol graphs were plotted and ID_{50}/IC_{50} values were calculated using non-linear regression analysis via GraphPad Prism.

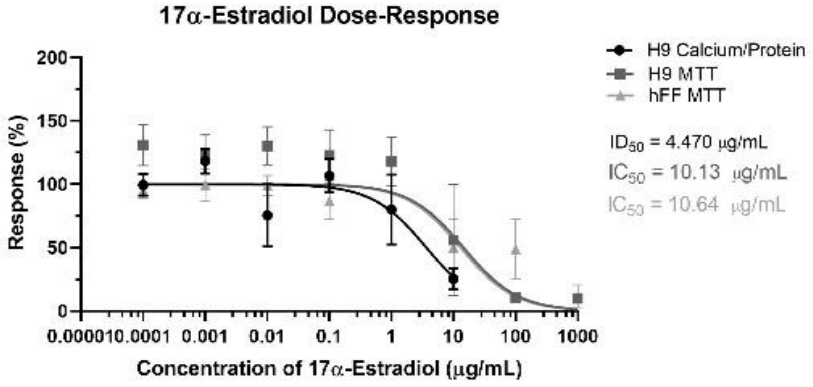

Figure 6: Human Embryonic Stem Cell Model - 17a-Estradiol graphs were plotted and ID_{50}/IC_{50} values were calculated using non-linear regression analysis via GraphPad Prism.

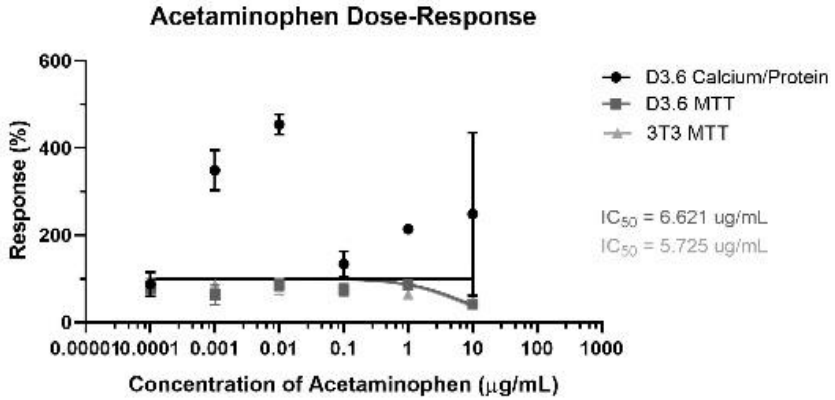

Figure 7: Mouse Embryonic Stem Cell Model - Acetaminophen graphs were plotted and ID_{50}/IC_{50} values were calculated using non-linear regression analysis via GraphPad Prism.

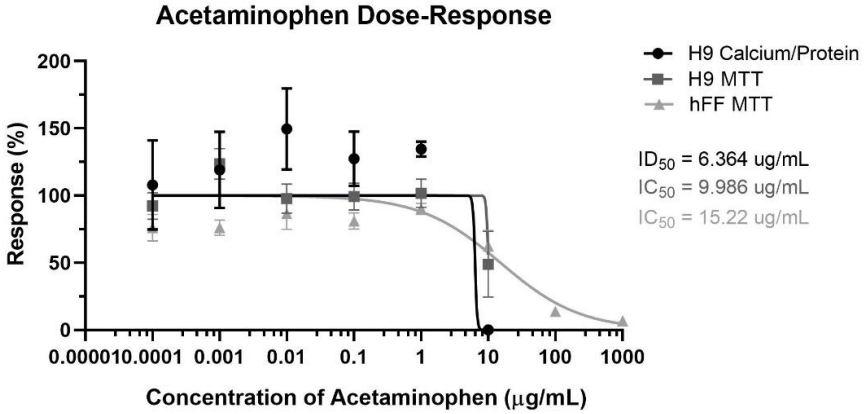

Figure 8: Human Embryonic Stem Cell Model - Acetaminophen graphs were plotted and ID_{50}/IC_{50} values were calculated using non-linear regression analysis via GraphPad Prism.

Discussion

All of the chemicals tested in this preliminary test set showed some detrimental response to the chemical over the period of the exposure. Determining the differences between the IC_{50} and the ID_{50} allowed us to measure the amount of calcification present and whether the detected differentiation inhibition was driven by the cytotoxicity of the chemical or whether it was independent from it. Viability and pluripotency are major aspects of ESCs, which essentially measure the cells ability to flourish under certain conditions. If this viability is threatened the cells will die which causes major damage to the development of higher level functioning, such as the formation of tissues and organs. By using an ESC model to analyze osteoblast formation, a new model can better predict and represent human regulatory chemicals in our body rather than use the outdated mouse model (Buck et al., 2018). The comparisons between both models allows us to predict which test better mimics the detrimental effects these chemicals can have on the mother and the fetus. Since IC_{50} measures cell viability and ID_{50} measures cell differentiation at 50% calcification, a low ID_{50} and a high IC_{50} could indicate that potential bone deformities might arise in the fetus (Kleinstreuer et al., 2011) . Furthermore, this translates to low differentiation, meaning the cells are not phenotypically and functionally changing with a high cell viability, stating that the cells are replicating. Eventhough the cells are alive and replicating, cells are failing to differentiate into specialized cells that are vital for development. Conversely, a high ID_{50} with a low IC_{50} indicates that the cells are dying but are differentiating. This combination also causes musculoskeletal malformations as the cells are dying. Despite the high rates of calcification, the cells are dying which negates the purpose of cell growth.

In the Coumarin dose-response curve, the mESC model had a greater response percentage when comparing the calculated IC_{50} readings. The slope of the graph was gradually

decreasing, though not completely visible, at the end of the exposure on day 20. Comparatively in the human model, Coumarin has a sharp decrease at around 0.01 µg/mL of the chemical (Figure 3), which might indicate the loss of differentiating capabilities at this particular concentration. Despite the cells still being alive under slightly higher concentrations, 0.01 µg/mL is a significant concentration in Coumarin where there is a loss of osteogenesis regardless of cell viability (Figure 4). This result allowed us to speculate that around the same concentration of exposure around pregnant mothers, this chemical has the potential to cause damage to fetal development (Wang et al., 2013). Exposure to this chemical may come in a multitude of ways, but since Coumarin is primarily found in cinnamon, overexposure may cause detrimental effects to the mother and the child during pregnancy. When looking at the dose-response curves of Coumarin, we can see a drastic difference in both the mouse and human models that is directly associated with the sensitivity between Coumarin and the type of ESC tested. In the mouse model, even though the concentrations were in higher dosage, there was no visible ID_{50} curve which helps us picture the loss of differentiation capacity in the mouse model. This lack of an ID_{50} illustrates the lack of sensitivity to Coumarin under the same concentrations tested in the human ESC model, where we can see a drastic decrease in the ID_{50} value to around 0.01 µg/mL (Figure 4).

Similar to Coumarin, 17α-Estradiol in the mouse model lacks the ID_{50} which is seen in the hESC model when tested at the same concentrations. This highlights the need for a species-relevant screening technique to determine human predictability. However, there is a notable constant line for the response percentage in relation to increases in the concentrations of 17α-Estradiol (Figure 5). In the hESCs, cell viability once again outlasts the capacity of cell differentiation, indicative of premature bone deformations when exposure to 17α-Estradiol

reaches a critical levels of exposure at about 4 µg/mL (Figure 6). Since 17α-Estradiol is found in many topical hair loss solutions, there can be an approximate prediction of the percentage of 17α-Estradiol that is safe for application during pregnancy (Thomas et al., 1983). The concentration-response curve for Acetaminophen was also very similar to the response percentage seen with Coumarin and 17α-Estradiol in the mouse model. The constant slope in response to the changes in the concentrations of the chemical exposed (Figure 7) nullifies the extent to which this chemical actually has an effect on fetal development. The Acetaminophen dose-response curve in a hESC model better represents these drastic effects in which we can see the sudden drop in differentiating capacity at around 6 µg/mL (Figure 8). Around this concentration the viability also dropped dramatically which questions the effects of this drug on the mother during pregnancy. This visual change in the IC_{50} and ID_{50} values in the mouse model once again highlights this discrepancy which can be noted in both the models. Clearly, a more sensitive model, especially to those chemicals that can cause serious damage, is preferred to that of the traditional mouse model which lacks the appropriate sensitivity threshold that is vital in embryotoxicity testing. Since both the viability and the differentiation capability drop at around the same concentration, prolonged exposure to this concentration may cause neuromuscular abnormalities during bone formation in the fetus (Bauer et al., 2021). Similarly identified ID/IC_{50} concentrations indicate that the differentiation effect is likely dependent on cell viability. Even though acetaminophen is a very common drug found in oral painkillers, limited exposure to this drug may benefit the mother while keeping the growing child safe. The mESC dose-response curve for Folic Acid was also very similar to the other chemical exposures, where the gradual decrease in the response percentage was noted as the concentration of the chemical concentration was increased (Figure 1).

The hESCs also allowed us to take a deeper look into the specific concentration at which folic acid exposure can cause permanent damage to the growing cells of the embryo. As seen with the other chemicals, the folic acid dose-response curve in hESC dropped drastically in differentiation capacity at around 6 µg/mL (Figure 2). The cells were supposedly viable after this concentration but they failed to differentiate properly into osteoblasts, suggesting such exposure could potentially cause bone irregularities *in vivo*. Since folate, the metabolized form of folic acid, is predominant in prenatal supplements, the mother is placed under heavy detriment (Ostrea et al., 2022). Overdosing on these prenatals or improper concentration restrictions of folate in these nutritional supplements can be seen to drastically decrease differentiation, posing a serious threat to the developing fetus.

Taken together, the routine use of an *in vitro* assay based on human ESCs might help researchers emulate the conditions to which these chemicals might influence osteogenic differentiation capacity of cells that develop in the fetus. Compared to the mouse *in vitro* assay, the greater applicability to human health encourages the scientific pursuits for safer treatment methods for fetal development. Using a human *in vitro* model is essentially a notable model to assess chemical exposure with greater predictability to human health and development over the standard mouse model screenings. Chemical sensitivity is a vital aspect to embryotoxicity screens, especially if small changes in concentrations may alter the musculoskeletal formation in the fetus. As clearly noted in the concentration-response curves, the mouse cells have a higher sensitivity threshold than the one present in the human ESCs, which calls to question the applicability of the mouse model over the use of hESCs to human health screenings. Limitations to this study can be associated with improper treatment of the cells to the various chemical exposures. These limitations, along with experimentator error, might be combated with proper

investigation into laboratory technique and management of ESCs. Ultimately, in addition to finding an ideal model system, exploring the health detriments of overexposure to certain chemicals in our environment can help mitigate developmental maladies targeting the mother and their unborn child.

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