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Developmental toxicity assessment of common excipients using a stem cell-based *in vitro* morphogenesis model

Chloe J. Yuan and Yusuke Marikawa

University of Hawaii John A. Burns School of Medicine, Department of Anatomy, Biochemistry and Physiology, Institute for Biogenesis Research, Honolulu, Hawaii, 96813

Abstract

Various chemical compounds can inflict developmental toxicity when sufficiently high concentrations are exposed to embryos at the critical stages of development. Excipients, such as coloring agents and preservatives, are pharmacologically inactive ingredients that are included in various medications, foods, and cosmetics. However, concentrations that may adversely affect embryo development are largely unknown for most excipients. Here, the lowest observed adverse effect level (LOAEL) to inflict developmental toxicity was assessed for three coloring agents (allura red, brilliant blue, and tartrazine) and three preservatives (butylated hydroxyanisole, metabisulfite, and methylparaben). Adverse impact of a compound exposure was determined using the stem cell-based *in vitro* morphogenesis model, in which three-dimensional cell aggregates, or embryoid bodies (EBs), recapitulate embryonic processes of body axis elongation and patterning. LOAEL to impair EB morphogenesis was 200 μ M for methylparaben, 400 μ M for butylated hydroxyanisole, 600 μ M for allura red and brilliant blue, and 1,000 μ M for metabisulfite. Gene expression analyses of excipient-treated EBs revealed that butylated hydroxyanisole and methylparaben significantly altered profiles of developmental regulators involved in axial elongation and patterning of the body. The present study may provide a novel *in vitro* approach to investigate potential developmental toxicity of common excipients with mechanistic insights.

Keywords

Developmental toxicity; In vitro morphogenesis; Gastrulation; Excipient; Butylated hydroxyanisole; Methylparaben

1. Introduction

Development of embryos in the reproductive tract can be disrupted by various environmental factors, which results in their malformation or death. Women of childbearing age may be

Corresponding author: Yusuke Marikawa, 651 Ilalo Street, Biosciences Building, 163A, Honolulu, HI 96813, Tel: (808) 692-1411, Fax: (808) 692-1962. marikawa@hawaii.edu.

Conflict of interest

There are no conflicts of interest regarding this manuscript, as confirmed by all authors.

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exposed to such developmentally toxic compounds through medications or environmental pollutants. The extent and nature of developmental impact depend on the physicochemical properties of a compound, dosage, and timing of exposure during or even before gestation (Daston et al., 2010; Friedman, 2010; Jelínek, 2005; Schardein and Macina, 2006). Developmental toxicity of pharmaceutical chemicals is typically investigated during the preclinical phase of research using model animals, such as rats and rabbits. In comparison, most of the “non-pharmaceutical” chemicals, including herbicides, pesticides, cosmetics, dietary supplements, industrial byproducts, and excipients (see below), are less regulated and are not as vigorously studied for developmental toxicity. Ideally, all chemical compounds should be assessed for developmental toxicity to minimize the incidence of fetal loss and birth defects. However, animal-based toxicity tests are generally expensive, laborious, time-consuming, and subject to animal welfare concerns. These barriers altogether hinder the facilitated regulation of the non-pharmaceutical chemicals. Thus, non-animal alternatives are strongly desired to test the developmental toxicity of a large number of compounds in an economical and speedy manner.

Pluripotent stem cells are cultured cell lines that retain the developmental potential of early embryos. These cells can be induced to differentiate into various cell types *in vitro*, including cardiomyocytes, neurons, and osteoblasts. Because of their embryo-like properties, pluripotent stem cells have been explored as non-animal alternatives to study developmental toxicity. In assays commonly referred to as embryonic stem cell tests (ESTs), the potential developmental toxicity of compounds is evaluated based on their inhibitory effects on the *in vitro* differentiation of ES cells as well as their cytotoxic impact on ES cell and somatic cell lines, specifically NIH/3T3 fibroblast (Riebeling et al., 2012; Theunissen and Piersma, 2012). The original EST, or ESTc, measures concentrations of a test compound that inhibit cardiomyocyte differentiation by 50% (ID50) as well as concentrations that inhibit proliferation of ES and NIH/3T3 cells by 50% (D3 IC50 and 3T3 IC50, respectively). ID50, D3 IC50, and 3T3 IC50 are then computed using a biostatistical model to predict potential developmental toxicity of the compound (Spielmann et al., 1997). ESTc performed well in the initial validation study against a series of compounds that are designated as strong, weak, or non-embryotoxic (Genschow et al., 2002). However, ESTc yielded a poor result in a follow-up study with additional reference compounds (Marx-Stoelting et al. 2009; Riebeling et al., 2012). Moreover, a significant concern has been raised regarding the designation of reference compounds as “toxic” or “non-toxic”, because developmental toxicity of a compound is conditional and depends on the concentration and timing of exposure (Daston et al., 2010, 2014).

In another type of stem cell-based assay, morphogenesis of P19C5 cell aggregates is used as readouts to evaluate developmental toxicity. P19C5 is a stem cell line originated from mouse embryonal carcinoma, and its three-dimensional culture generates cell aggregates, or embryoid bodies (EBs), that exhibit morphogenesis reminiscent of gastrulation (Lau and Marikawa, 2014; Marikawa et al., 2009). Gastrulation is the fundamental step of embryogenesis that creates the three germ layers and drives axial elongation and patterning of the body (Stern, 2004). During four days of culture, P19C5 EBs grow in size and transform from a spherical to elongated shape that possesses a polarity corresponding to the anterior-posterior body axis. Expressions of various developmental regulator genes are

temporally and spatially controlled during *in vitro* development of P19C5 EBs in a manner comparable to embryogenesis at the gastrulation stages (Lau and Marikawa, 2014; Li and Marikawa, 2015; Marikawa et al., 2009). Growth, elongation, and temporal and spatial gene expression profiles are significantly altered in EBs by pharmacological inhibitors of signaling pathways essential for embryo patterning (Li and Marikawa, 2015) and by human medications that are contraindicated for use during pregnancy (Li and Marikawa, 2016; Warkus et al., 2016). Recently, the morphogenesis-based assay using P19C5 EBs has been validated against a list of reference compound exposures compiled by Daston and colleagues (Daston et al., 2014), which consists of specific *in vivo* concentrations of various compounds that exhibit developmental toxicity or lack of it. The study indicates that *in vitro* impact on EB morphogenesis is largely in line with *in vivo* developmental effects with concordance of 71.4 to 82.9 %, when adverse impact is defined as significant alterations in quantifiable morphometric parameters that signify the size and shape of EBs (Warkus and Marikawa, 2017). Thus, the morphogenesis of EBs can serve as an effective endpoint of analysis to assess whether a certain compound at a particular concentration would exhibit developmental toxicity.

The objective of the present study is to evaluate developmental toxicity of common excipients using the *in vitro* morphogenesis of P19C5 EBs. Excipients are the pharmacologically inactive ingredients, such as colorings, preservatives, and fillers, which are included in medications, foods, drinks, and cosmetics. Many excipients are classified by the United States Food and Drug Administration (FDA) as generally-recognized-as-safe (GRAS) substances, based on scientific evidence (Burdock and Carabin, 2004; Neltner et al., 2013; Roberts and Haighton, 2016; Osterberg and See, 2003). The guidance documents issued by the FDA for the safety evaluation of food ingredients and pharmaceutical excipients include guidelines for developmental toxicity studies using model animals (US FDA, 2005, 2007). However, these guidelines are essentially recommendations without strict regulations, and thus the data on developmental toxicity are relatively scarce for many excipients. Furthermore, unlike pharmaceutical drugs, human epidemiologic studies are ineffective in linking particular excipients with occurrence of birth defects or miscarriage, partly because most people are regularly exposed to a number of excipients without detailed records or recollections about types, dosages, and frequencies of exposures. Thus, it would be beneficial to gain further information on individual excipients, such as dose-response relationship with respect to developmental toxicity.

Six excipients were evaluated in this study, three of which are commonly used as coloring agents (allura red AC, brilliant blue FCF, and tartrazine) and the others as preservatives (butylated hydroxyanisole, metabisulfite, and methylparaben). First, dose-response relationships for effects of each excipient on EB morphogenesis were determined. Second, impact on gene expression profiles was analyzed for the two excipients, butylated hydroxyanisole and methylparaben, which affected EB morphogenesis most potently among the six excipients. Expressions of 16 genes, encoding transcription factors and signaling molecules that regulate the key events of gastrulation, were specifically evaluated in relation to the morphogenetic effects of the excipients (Table 1). The data provided by the present study serve as a foundation for future investigations on the potential developmental toxicity inflicted by exposures to these common excipients.

2. Materials and methods

2.1. Test compounds

All test compounds were obtained commercially (Sigma-Aldrich, St Louis, MO), namely allura red AC (#458848), brilliant blue FCF (#80717), tartrazine (#86310), butylated hydroxyanisole (#B1253), sodium metabisulfite (#08982), and methylparaben (#PHR1012). The chemical structures of the test compounds are shown in Fig. 1A. The first four compounds were dissolved in water and the latter two compounds were dissolved in dimethyl sulfoxide (DMSO) at 100 mM as stocks and stored at -20°C .

2.2. Cell culture

P19C5 cells (Lau and Marikawa, 2014) were propagated in culture medium (Minimum Essential Medium Alpha with nucleosides and GlutaMAX Supplement [Life Technologies, Carlsbad, CA], 2.5% fetal bovine serum, 7.5% newborn calf serum, 50 units/mL penicillin, and 50 $\mu\text{g/mL}$ streptomycin). EBs were generated according to the method previously described (Marikawa et al., 2009). Briefly, P19C5 cells were fully dissociated with Trypsin-EDTA, and suspended in culture medium containing 1% DMSO at the density of 10 cells/ μL with or without specific amount of a test compound (Fig. 1B–D). Drops (20 μL each) of cell suspension were spotted on the inner surface of Petri dish lids for hanging drop culture. Note that excipient stocks are prepared in either water or DMSO, as described above (2.1), and all control and excipient-treated EBs were cultured in the presence of DMSO adjusted to the final concentration of 1%, regardless of the type of vehicle used in the stocks.

2.3. Measurement of morphometric parameters

EBs were removed from hanging drops and grouped together for photography using an AxioCam MRm digital camera connected to an Axiovert 200 microscope with Hoffman modulation-contrast optics (Carl Zeiss, Thornwood, NY). Image files were converted to JPEG format and opened in the ImageJ program (<http://rsb.info.nih.gov/ij>). Morphological parameters of individual Day 4 EBs, namely area and circularity ($= 4 \times \pi \times \text{area} / \text{perimeter}^2$), were measured on ImageJ by manually tracing their circumference using the polygon selection tool. – Circularity was converted to Elongation Distortion Index ($\text{EDI} = 1 / \text{circularity} - 1$), as it is more reflective of the extent of EB elongation (Marikawa et al., 2009). As described previously, area was used as a proxy for the size of EB, whereas EDI was used to gauge the extent of EB axial elongation (Marikawa et al., 2009; Warkus et al., 2016).

2.4. Quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from EBs using TRI reagent (Sigma-Aldrich) and Direct-zol RNA MiniPrep kit (Zymo Research, Irvine, CA) and processed for cDNA synthesis using M-MLV Reverse Transcriptase (Promega, Madison, WI) and oligo-dT primer. Quantitative PCR was performed using the CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA) with iQ SYBR Green Supermix (Bio-Rad) as follows: initial denaturation at 94°C for 5 min, followed by up to 45 cycles of 94°C for 15 sec, 60°C for 20 sec, and 72°C for 40 sec. Data files were opened in CFX Manager software (Bio-Rad) and Ct values were transferred to the

Excel program for further analyses. *Actb*, which encodes β -Actin, was used as a housekeeping gene to normalize the expression levels of other genes. Sequences of the primers used are shown in Table 1.

2.5. Statistical analysis

Experiments to assess morphological impact of compound exposures on EB morphology were conducted in three biological replicates (for the concentration series of 10, 100, and 1,000 μ M) or duplicates (for the concentration series of 200, 400, 600, and 800 μ M) using different collections of cell suspensions. For each replicate, 16 hanging drops were generated per exposure (a specific concentration of compound) in parallel with 16 control (i.e., no compound) hanging drops (Fig. 1D). Note that the average area and EDI of control EBs were slightly different among replicates. Thus, normalization was performed against the control values in each replicate to yield relative area and relative EDI of compound-treated EBs. Relative area and relative EDI data from all replicates were compiled, and their averages are shown with 95% confidence intervals. To verify that the observed effects on EBs were statistically significant, two-sample t-test was performed between compound-treated group and the control group. All morphological impacts that were defined as adverse in the present study (see below) were statistically significant ($P < 0.01$). Gene expression analyses were conducted using three independent sets of samples as biological replicates. Each set consisted of 9 samples: Day 0 (dissociated cells immediately before aggregation), control EBs at Days 1 to 4, and compound-treated EBs at Days 1 to 4, all of which were originated from the same cell suspension. Relative expression levels were calculated for each set of experiment, and the averages of the three replicates are shown with standard deviations. Two-sample t-test was performed between control and compound-treated groups to determine significant differences in relative expression levels ($P < 0.05$).

2.6. Definition of adverse morphogenetic impact

A compound exposure was defined as having an “adverse” effect on EB morphogenesis when it caused either of the following three outcomes: (1) degeneration (i.e., death) of EBs at any time point in culture or cell aggregation failure, (2) a reduction in the average area by more than 20% relative to control EBs, and (3) a decrease or increase in the average EDI by more than 40% relative to control EBs, according to the previous validation study (Warkus and Marikawa, 2017).

3. Results

3.1. Morphogenetic impact of excipients on embryoid bodies

In the initial series of experiment, six excipients were evaluated at three concentrations (10, 100, and 1,000 μ M) for their impact on P19C5 EB morphogenesis. Two quantifiable morphometric parameters, namely area and EDI, of Day 4 EBs were compared between excipient-treated and control groups (Fig. 1D). Significant differences in relative area or relative EDI were defined as adverse effects caused by an excipient exposure, according to the criteria described in Materials and methods (2.6). No adverse effect was observed for any of the excipients at 10 or 100 μ M. However, at 1,000 μ M, five excipients adversely affected EB morphogenesis (Figs. 2 and 3). Exposure to allura red AC impeded growth and

elongation of EBs, as shown by reductions in relative area and relative EDI. Likewise, brilliant blue FCF and methylparaben reduced relative area and relative EDI. On the other hand, metabisulfite caused a reduction in EDI but not in area, resulting in a shorter but wider EB morphology (Fig. 3). Exposure to butylated hydroxyanisole caused degeneration of EBs.

Additional concentrations between 100 and 1,000 μM were further evaluated to determine the lowest observed adverse effect level (LOAEL) for each excipient. LOAEL that affected EB morphogenesis was 600 μM for allura red AC (reduced area), 400 μM for butylated hydroxyanisole (reduced area), 600 μM for brilliant blue FCF (reduced area), and 200 μM for methylparaben (increased EDI). Metabisulfite affected EB morphogenesis only at 1,000 μM , whereas no adverse effect was observed for tartrazine at all concentrations evaluated. The results are summarized in Table 2.

3.2. Impact of excipients on gene expression profiles of developmental regulators

As shown in previous studies, various developmental regulator genes exhibit distinct expression patterns during the course of EB development, and their expression profiles are significantly altered by exposures to pharmacological inhibitors of developmental signals (Lau and Marikawa, 2014; Li and Marikawa, 2015; Marikawa et al., 2009). Thus, the impact of excipients on gene expression profiles was analyzed to provide molecular readouts of their morphogenetic effects. The two excipients, butylated hydroxyanisole (BHA) and methylparaben (MP), were specifically examined in the present study, as they affected EB morphogenesis most potently among the six excipients.

Temporal expression patterns of 16 developmental regulator genes (Table 1) were compared between control and excipient-treated EBs. Effects of BHA and MP were analyzed at 600 μM and 400 μM , respectively. These concentrations were chosen for analyses, because they were the lowest concentrations that altered both morphometric parameters of EBs, i.e., area and EDI, and may provide molecular insights that are relevant to both morphological alterations. For the BHA treatment, some genes were largely unaffected, whereas others were significantly altered. A pluripotency maintenance gene *Pou5f1* (also known as *Oct4*) was markedly down-regulated by Day 1 in control EBs, and a similar pattern of down-regulation was also observed in BHA-treated EBs (Fig. 4). In contrast, expression patterns of transcription factor genes that regulate mesendoderm specification, namely *Brachyury*, *Cdx1*, *Mixl1*, *Sp5* and *Lhx1*, were significantly affected by BHA. These genes were strongly up-regulated by Day 1 in control EBs, whereas their Day 1 up-regulations were all diminished in BHA-treated EBs. The genes involved in embryo elongation and patterning at the caudal end, specifically *Wnt3a*, *Tbx6*, *Hes7* and *Lfng*, were all strongly up-regulated at Day 2 in control EBs. The Day 2 peak expressions of *Tbx6* and *Lfng* were significantly diminished by BHA, whereas those of *Wnt3a* and *Hes7* were not. Expression of *Meox1*, which encodes a transcriptional regulator for somite segmentation, peaked at Day 3 in control EBs. However, the *Meox1* expression was essentially abrogated by BHA treatment. Hox genes, regulators of axial patterning, were up-regulated during the course of EB development in a gene-specific temporal manner, and were differentially affected by BHA. BHA treatment diminished *Hoxb9* and *Hoxa1* levels at Day 3 and 4, respectively, while it did not significantly alter *Hoxc6*. *Aldh1a2*, a regulator of retinoic acid production, exhibited

robust up-regulation at Day 4 in control EBs, which was significantly diminished in BHA-treated EBs. Lastly, *Pax3*, a transcriptional regulator for myogenesis and neural patterning, was gradually up-regulated during the course of EB development, which was essentially unaffected by BHA treatment. These results indicate that BHA influenced temporal expression profiles in a gene-specific manner, so that some genes were more severely affected whereas others were not.

MP also differentially altered gene expression profiles. While the morphogenetic impact was distinct, many genes were not largely affected by MP treatment, including *Pou5f1*, *Brachyury*, *Mixl1*, *Lhx1*, *Wnt3a*, *Tbx6*, *Hes7*, *Lfng*, *Hoxb9*, and *Hoxa1* (Fig. 5). The most dramatic alteration caused by MB treatment was abrogation of the Day 3 peak expression of *Meox1*, which was also observed in BHA-treated EBs. The Day 4 up-regulation of *Aldh1a2* was diminished by MP, also similar to the BHA treatment. In contrast, the effects on *Cdx1*, *Sp5*, *Hoxc6*, and *Pax3* expression profiles were different between MP and BHA treatments. The Day 1 peak expressions of *Cdx1* and *Sp5* were further enhanced in MP-treated EBs, unlike the case in BHA-treated EBs. Also, the Day 3 expressions of *Hoxc6* and *Pax3* were significantly elevated by MP, which were not observed for BHA.

4. Discussion

Many people, including women of childbearing age, are unknowingly exposed to various excipients through foods and medications. For example, the National Health and Nutrition Examination Survey shows that more than 50% of the general population has been exposed to paraben compounds, such as methylparaben and butylparaben (US CDC, 2015). Any compounds can be developmentally toxic when sufficiently high amounts are exposed to embryos at the critical stages of development, and therefore, it is impractical to assign a given compound as a developmental toxicant or not (Daston et al., 2010; Friedman, 2010; Jelínek, 2005). It is more important to ensure that the systemic concentration of a given compound in women of childbearing age is kept far below its LOAEL, i.e., the lowest concentration that causes an adverse impact on developing embryos. In the present study, LOAEL for developmental toxicity was evaluated for six common excipients using the P19C5 EB morphogenesis assay, the effectiveness of which has recently been demonstrated using the exposure-based validation list (Warkus and Marikawa, 2017). Five of the 6 excipients tested displayed adverse effects at the LOAEL of 200 μ M (methylparaben [MP]), 400 μ M (butylated hydroxyanisole [BHA]), 600 μ M (allura red AC and brilliant blue FCF), and 1,000 μ M (metabisulfite) (Table 2). These concentrations may be considerably higher than what most people are exposed to. For example, the median concentration of MP in urinary samples collected from a demographically diverse group of 100 adults is 43.9 ng/mL or about 0.29 μ M (Ye et al., 2006), and the concentration of MP in urine is considered to be much higher than serum (Teitelbaum et al., 2016). Thus, the result of the present study does not necessarily raise a significant concern for potential developmental toxicity from casual intake of these excipients.

Nonetheless, the systemic concentrations of excipients may exceed their LOAEL under certain conditions. For example, atypical dietary habits can predispose to excessive intake of foods that contain preservatives or colorings. Also, the systemic concentrations of excipients

may be dramatically elevated in individuals depending on health conditions (e.g., liver or kidney dysfunction that impair compound elimination), genetic variations (e.g., poor metabolizers), and concomitant intake of other medications that inhibit metabolizing enzymes (Lynch et al., 2007). Furthermore, some excipients have been shown to exhibit synergistic effects with other compounds to cause toxicity. For example, a combination of food colorings, such as brilliant blue FCF and tartrazine together, suppresses proliferation of neural progenitor cells, leading to impaired neurogenesis (Lau et al., 2006; Park et al., 2009). BHA has been shown to enhance the effect of carcinogens, such as N-methylnitrosourea (MNU) and dimethyl-4-aminobiphenyl (DMAB), in model animals (Ito et al., 1989; Shirai et al., 1991). Therefore, continued research on excipients is important to monitor systemic concentrations in a wide range of population and to elucidate confounding factors that may exacerbate their potential developmental toxicity.

Effects on gene expressions are generally considered as sensitive endpoints to assess the developmental toxicity of compounds. Accordingly, various types of *in vitro* tests have incorporated gene expression analyses to enhance their performance (Buesen et al., 2009; Gao et al., 2014; Panzica-Kelly et al., 2013; Pennings et al., 2011; Suzuki et al., 2011; Yu et al., 2015; zur Nieden et al., 2001). In the present study, the impact of BHA and MP was evaluated based on the expression patterns of 16 genes that encode regulators of germ layer formation and axial patterning (Table 1). However, it is likely that the excipient treatments affected other genes that are also developmentally important. In future, more extensive gene expression analyses with high throughput technologies, such as RNA-seq, should yield comprehensive transcriptomic views on how excipients may interfere with embryogenesis at the molecular levels.

The P19C5 EB developmental toxicity assay is based on impact of chemical exposure on morphogenesis, i.e., collective cell movement to construct the 3-dimensional shape of tissues. Many common birth defects are caused by misregulation of morphogenesis, such as neural tube closure defects, heart septal defects, congenital diaphragmatic hernia, hypospadias, and cleft lip and palate (Sadler, 2016). Thus, morphogenesis-based assays can provide an embryologically relevant context to investigate developmental toxicity of chemical exposures that are linked to birth defects. Gene expression profiles suggest that the *in vitro* development of P19C5 EBs represents early stages of post-implantation development. The EB morphogenesis is controlled by various developmental signals, such as Wnt, Nodal, Fgf, Bmp, and retinoic acid signaling (Li and Marikawa, 2015). All of these developmental signals are also crucial for later stages of development, including the maturation of various organs. Thus, the morphogenesis of P19C5 EBs may also serve as an effective assay for a wide range of compound exposures that have adverse effects on embryos throughout development.

One of the weaknesses of *in vitro* assays, in general, is the lack of maternal metabolism. Some compounds exert developmental toxicity only after metabolic modifications by the mother. Such compounds, in theory, cannot be detected by *in vitro* assays that only represent embryological processes. The P19C5 EB morphogenesis has failed to detect the developmentally toxic exposure of methanol in the validation study (Warkus and Marikawa, 2017), which may be due to its inability to metabolize methanol into its toxic derivative,

formic acid. Although the present study established LOAEL for the six excipients, it is possible that their metabolites may exert adverse impact at lower concentrations. Future endeavors are crucial to incorporate a metabolic system, such as hepatocytes or liver extracts, to augment the detection capability of the *in vitro* assay.

5. Conclusions

Women of childbearing age are exposed to a large number of non-pharmaceutical compounds, including excipients, which are often not strictly regulated nor monitored. More information for each non-pharmaceutical compound is desired, specifically the LOAEL that exerts potential developmental toxicity, to help improve chemical regulatory guidelines. In the present study, a stem cell-based *in vitro* morphogenesis assay was employed to determine LOAEL of the common food colorings and preservatives that causes significant alterations in morphology and gene expression patterns. Further studies are warranted to ensure that embryos are not exposed to a high level of these excipients that may impair critical developmental processes.

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Highlights

- In vitro morphogenesis model is used to assess developmental toxicity of excipients
- 6 common excipients, namely 3 coloring agents and 3 preservatives, are evaluated
- Methylparaben and butylated hydroxyanisole disturbed morphogenesis most potently
- Gene expression profiles are altered by methylparaben and butylated hydroxyanisole
- More studies are needed to determine LOAEL of developmental toxicity for excipients

Fig. 1. Morphogenesis of P19C5 embryoid bodies

A. The chemical structures of the test compounds. **B.** The Formation of cell aggregates, or embryoid bodies (EBs), in hanging drops of culture medium. **C.** Representative images of unmanipulated EBs to demonstrate the time course of morphological changes over four days of culture. **D.** Experimental scheme to assess morphogenetic impact of compound exposures.

Allura red AC **Butylated hydroxyanisole** **Brilliant blue FCF** **Metabisulfite** **Tartrazine** **Methylparaben** **Fig. 2. Impact of the excipients exposures on P19C5 EB morphogenesis**

For each compound, concentrations tested are indicated in the top row of the table with a summary of observed morphogenetic impact on EB area and EDI, indicated with upward arrowheads (increase) and downward arrowheads (reduction). No area or EDI value is available when EBs were dead (D). Column graphs below the summary tables show averages of relative area (white columns) and relative EDI (gray columns), for the corresponding compound concentrations indicated above. Error bars show 95% confidence intervals. Asterisks indicate adverse impacts, which are defined in the present study as a change in average area by >20% or a change in average EDI by >40% relative to controls. Numbers at the bottom (N) are numbers of EBs scored.

Fig. 3. Morphogenetic impact of excipients on P19C5 EB

Representative images of EBs. Each set of images shows a control group of EBs (no test compound; 0 μM) and one or two compound-treated groups of EBs, made from the same cell suspension as the control. Morphogenetic effects, as presented in Fig. 2, are indicated below each compound-treated EB image for reference. Scale bars = 500 μm.

Fig. 4. Impact of butylated hydroxyanisole on gene expression patterns

Quantitative reverse-transcriptase PCR analyses of developmental regulator gene expression over the culture period. Horizontal axes represent days of culture while vertical axes represent relative expression levels, in arbitrary units. Blue and red lines correspond to the relative expression levels (mean $\pm$ standard deviation; $n = 3$) in control EBs and EBs treated with 600 μ M butylated hydroxyanisole (BHA), respectively. Asterisks indicate significant reduction or increase ($p < 0.05$; 2-sample t-test) in mean relative expression levels by BHA treatment on a given day of EB culture, as compared to the control.

Fig. 5. Impact of methylparaben on gene expression patterns

Quantitative reverse-transcriptase PCR analyses of developmental regulator gene expression over the culture period. Horizontal axes represent days of culture while vertical axes represent relative expression levels, in arbitrary units. Blue and green lines correspond to the relative expression levels (mean $\pm$ standard deviation; n = 3) in control EBs and EBs treated with 400 μ M methylparaben (MP), respectively. Asterisks indicate significant reduction or increase (p < 0.05; 2-sample t-test) in mean relative expression levels by MP treatment on a given day of EB culture, as compared to the control.

Table 1

Genes examined in the present study

Gene Name	Characteristics	Primer Sequences (5' → 3')
Actb	Cytoskeletal actin; House-keeping; Ubiquitously expressed	F: GAGAGGGAAATCGTGCGTGACATC R: CAGCTCAGTAACAGTCCGCCTAGA
Aldh1a2	Retinoic acid synthesis; Axial patterning	F: CTTGCCTCACAACAAGTGAGCTTC R: TCACCCAGGTTAGAGACTGGCTTC
Brachyury	T-box transcription factor; Mesendoderm specification	F: CCTCGGATTCACATCGTGAGAGTT R: AGTAGGTGGGCGGGCGTTATGACT
Cdx1	Homeodomain transcription factor; Mesendoderm specification	F: TCAGGACTGGACATGAGGTAGAGG R: TGGGAAGGTGGGCATGAGCAGGTA
Hes7	bHLH transcription factor, Somite segmentation	F: CATACCCTTCTCCCACCTTTAGGC R: AGTGACGAGAAAGCGATTCAAAGG
Hoxa1	HOX gene (anterior group); Axial patterning	F: CCCTTTCCTTCCACACTGTCTTGT R: AAGACCCGTAAACTCTGCTCTGGA
Hoxb9	HOX gene (posterior group); Axial patterning	F: AAGCAGGGAGTGGTTTTATGAAGG R: GGGATAGGAATGTATGAATGGGGA
Hoxc6	HOX gene (central group); Axial patterning	F: TTCGCCACAGGAGAATGTCGTGTT R: CGAGTTAGGTAGCGGTTGAAGTGA
Lfng	Notch signaling regulator; Somite segmentation	F: TGCTAGCACCTATCTGGAGCCTTC R: TGGGCGACTTCTCTCTACTTTGG
Lhx1	Homeodomain transcription factor; Mesendoderm specification	F: TCGGCTCACTGTGCTAGTATGTA R: AACACTTCTCAGGTTGCTGGTGC
Meox1	Homeodomain transcription factor; Somite differentiation	F: AAAAATCAGACTTCCCAGCGACAG R: TTCACACGTTTCCACTTCATCCTC
Mixl1	Homeodomain transcription factor; Mesendoderm specification	F: CGACAGACCATGTACCCAGACATC R: TGAGGCTTCAAACACCTAGCTTCA
Pax3	Homeodomain transcription factor; myogenesis; neural patterning	F: GCTTCTCAGCGTGCAATACTGTGT R: TTTCTGTTCTAGCCCTGCCTTTTG
Pou5f1	POU domain transcription factor; Pluripotency maintenance	F: AGGCAGGAGCACGAGTGGAAGCA R: GGAGGGCTTCGGGCACTTCAGAAA
Sp5	Zinc finger transcription factor; Mesendoderm specification	F: CAGGACAGGAACTGGGTCGTAGT R: GGCCTAGCAAAAACCTTAGGCCTTG
Tbx6	T-box transcription factor; Regulation of neuromesodermal progenitors	F: GGCCTCTCTCCACCTTTAGTTTC R: CACTAGTAACAAGGCCCCAGGAG
Wnt3a	Wnt signaling ligand; Regulation of neuromesodermal progenitors	F: GCCACAAGAGCTTCCTGATTGGTA R: CCAGGCAGAAGACAGTCAGTCACC

Table 2

Adverse impact of excipient exposures on EB morphogenesis

Compound	NOAEL	LOAEL	Morphogenetic effects*
Allura red AC	400 µM	600 µM	Reduced area
Brilliant blue FCF	400 µM	600 µM	Reduced area
Tartrazine	1,000 µM	ND	ND
Butylated hydroxyanisole	200 µM	400 µM	Reduced area
Metabisulfite	800 µM	1,000 µM	Reduced area
Methylparaben	100 µM	200 µM	Increased EDI

NOAEL, No-observed-adverse-effect-level; LOAEL, Lowest-observed-adverse-effect level; ND, Not determined; EDI, Elongation Distortion Index

* Morphogenetic effects observed at LOAEL