Original Article

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Effect of methyl p-hydroxybenzoate on the culture of mammalian cell

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Summary **Several chemicals, such as methyl p-hydroxybenzoate (MHB), have been widely used as preservatives in the water baths of CO_2 incubators used for mammalian cell culture, and they are not considered to produce any biological effects. However, no detailed analyses of the effects of these compounds on cultured cells have been reported. In this study, we thus examined whether MHB in the incubator water bath affects cell viability or genome-wide gene expression in mouse embryonic stem cells under control conditions [using only dimethyl sulfoxide (DMSO) in the culture medium] and under chemical-treated conditions using benzene and chloroform; conditions that simulate a cell-based toxicity assay. We found that (*i*) MHB significantly altered cell growth rate, and (*ii*) MHB affected gene expression levels related to pathways that modulate cell growth and basic molecular processes, not only under control conditions but also the chemical-treated conditions. Furthermore, Gene Ontology term analyses revealed that the effects of MHB cannot be accounted for by subtracting the gene expression pattern in the control conditions from that in the chemical-treated conditions. Thus, we suggest that the use of MHB or other preservatives in a CO_2 incubator water bath is reconsidered in terms of potential confounding effects on cultured cells.**

Keywords: Methyl p-hydroxybenzoate, mouse embryonic stem cells, deep sequencing; incubator, toxicological assessment

1. Introduction

In regular laboratory practice, mammalian cells are cultured in a CO_2 incubator using chemical compounds such as methyl p-hydroxybenzoate (MHB), sodium dehydroacetic acid, sodium dodecyl sulfate, ethylenediaminetetraacetic acid, sodium azide, or copper sulfate in the incubator water bath as a preservative to prevent bacterial or mycoplasma contamination. These chemicals are not considered to produce any biological effects on the cultured cells. For example, MHB, which is widely used for this purpose, was reported to be mostly non-toxic by both oral and parenteral routes, and non-irritating and non-sensitizing to normal skin at acute toxicity levels in animal experiments (*1*). However, no detailed analyses of *in vitro* cell responses to these chemicals have been reported. Cell stress responses are highly conserved responses to environmental changes with transient reprogramming of transcriptional, translational, and post-translational activities (*2*). Thus, culture conditions are critical for correct and reproducible studies using cultured cells.

In this study, we investigated the effect of MHB

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in the incubator water bath on the cell viability, and performed deep sequencing analyses (RNA-seq) on mouse embryonic stem cells (mESCs) cultured with MHB to determine the effects of MHB exposure on genome-wide gene expression. We chose mESCs because mESCs are basic cells and have a normal karyotype, maintain high telomerase activity, and exhibit remarkable long-term proliferative potential. We also examined two chemicals listed in the Japan Pollutant Release and Transfer Register as a class I designated chemical substances.

2. Materials and Methods

2.1. *Chemicals*

MHB, benzene, and chloroform were obtained from Wako, Osaka, Japan. MHB was used in a water bath. Benzene and chloroform were dissolved in dimethyl sulfoxide (DMSO) (Wako) and diluted in culture medium at a 0.1% vol/vol final concentration.

2.2. *Cell culture*

The mESC line H-1 was isolated from C3H/He mice by Kitani *et al*. (*3*). mESCs were maintained in Dulbecco's modified Eagle's medium (4.5 g/L glucose) with L-glutamine, without sodium pyruvate, (Nacalai Tesque, Kyoto, Japan) supplemented with 15% fetal bovine serum (Gibco, MA, USA), 1,000 U/mL Stem Sure Leukemia Inhibitory Factor (mouse, recombinant, solution; Wako), 0.1 mM Stem Sure 2-mercaptoethanol solution (Wako), and penicillin-streptomycin (Gibco). Cells were grown on mitomycin C (Kyowa Kirin, Tokyo, Japan)-treated mouse embryonic fibroblast feeder cells (C57BL/6J) at 37°C in a humidified incubator with 5% CO_2 without MHB. For the feeder-free culturing, mESCs were cultured in ESGRO complete plus serum-free clonal grade medium (Merck Millipore, Darmstadt, Germany) on gelatin (Sigma, MO, USA)-coated dishes without feeder cells at 37°C in a humidified incubator with 5% CO_2 without MHB for 4 days (pre-culture). In the following experiments, mESCs without feeder-free cells were used for chemical stress treatments. For chemical stress treatments, mESCs were cultured with or without 2% MHB (4 g in 200 mL sterilized water) in the incubator water bath, which is the concentration of MHB used commonly in our lab.

2.3. *Chemical stress treatments*

Cells without feeder cells (pre-culturing without MHB for 4 days) were seeded at 3.8×10^5 cells per well (6-well plate) in 2 mL medium or at 1.9×10^4 cells per well (96-well plate) in 100 µL medium. The cells were incubated overnight at 37°C with 5% CO_2 with or without MHB for 24 h. In separate analyses, cells were treated with 2 µL benzene (final concentration of 1,000 µM) or 2 µL chloroform (1,000 µM) for 24 h. Total RNA was extracted from cells in the 6-well plate with 400 µL RNAiso Plus (Takara, Kyoto, Japan) according to the manufacturer's instructions. The amount of total RNAs were analyzed using Nano Drop 2000c spectrophotometer (Thermo scientific, MA, USA). The numbers of viable cells in the 96-well plate were counted using a Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) in accordance with the manufacturer's instructions.

2.4. *RNA-seq and data analysis*

RNA-seq analyses were performed by Takara, Japan. First, ribosomal RNA was removed using a Ribo-Zero Magnetic Gold Kit (Human/Mouse/Rat) (Illumina, CA, USA) from 6 µg total RNA. An RNA-seq library was constructed using the TruSeq Standard mRNA Sample Prep Kit (Illumina). One hundred base pair-end read RNA-seq tags were generated using an Illumina HiSeq 2500 sequencer according to the standard protocol. Fluorescence images were processed to sequences using the analysis pipeline supplied by Illumina. RNA-seq tags were mapped to the mouse genome (hg19) from the National Center for Biotechnology Information using TopHat mapping software. Genic representations using fragments per kilobase of exon per million mapped fragments (FPKM) to normalize for gene length and depth of sequencing were calculated. RNA-seq tags were assigned to corresponding RefSeq transcripts when their genomic coordinates overlapped. We used RNA sequences available from public databases: mRNA from NM of RefSeq and lncRNA candidates from NR of RefSeq.

2.5. *Data access*

Short-read sequence archive data in this study are registered in GenBank (*http://www.ncbi.nlm.nih.gov/genbank*)/DDBJ (*http://ddbj.sakura.ne.jp*). The data to determine the expression levels of transcripts are registered as follows: accession nos. DRA005417; DRX076620, DRX076621, DRX076628, DRX076629, DRX076638, and DRX076638 (with MHB; MHB +), and DRX076650, DRX076651, DRX076658, DRX076659, DRX076668, and DRX076669 (without MHB; MHB –).

3. Results

3.1. *Effect of MHB on cell viability*

First, to examine the effect of MHB in the incubator water bath on the cell viability of cultured cells, mESCs were cultured with or without MHB for 24 h.

Figure 1 shows a phase-contrast image of the mESCs. No floating cells (dead cells) were observed under microscopic observation (data not shown). Moreover, the amounts of isolated RNAs from one well (6-well plate) in duplicate with or without MHB were 5.1 ± 0.3 or 5.8 ± 0.3 µg (mean ± errors, n = 2), respectively. Taken together, after culture with MHB, the number of mESCs was significantly decreased compared with the number of mESCs after culture without MHB.

3.2. *Effect of MHB on genome-wide gene expression*

To examine the effect of MHB on genome-wide gene expression patterns, we performed deep sequencing analyses (RNA-seq) on mESCs cultured with (MHB +) or without MHB (MHB –) for 24 h. We then analyzed more than 40 million RNA-seq tags from each sample. Genic representations were calculated using FPKM to normalize for gene length and depth of sequencing. Sequencing tags were then mapped to the mouse reference genome sequence using mapping software, allowing no mismatches. In total, 32,586 RNAs from the NM and NR categories of the Reference Sequence (RefSeq) Database were used for RNA annotation. The above examinations were also conducted for cultured cells under control conditions (DMSO only in the culture medium) and under benzene-treated conditions, and chloroform-treated conditions. This is a simplified simulation cell-based toxicity assay.

To quantify the effect of MHB on gene expression patterns, correlation coefficients of the logarithms of FPKMs were determined, both between MHB + and MHB –, and between replicated samples of MHB –, respectively, in the control conditions and in the chemical-treated conditions (the benzene- and chloroform-treated conditions). Figure 2 shows that the correlation coefficient between MHB + and MHB – was significantly lower than that of the replicated MHB – in the control conditions by approximately 0.1. A similar tendency was observed for the benzene-treated conditions (Figure 3) and for the chloroform-treated conditions (Figure 4). Moreover, cell viabilities were measured after exposure to 1,000 µM benzene and 1,000 µM chloroform for 24 h without MHB. Cell viabilities were decreased to 79 ± 13% and 66 ± 10% (mean ± SD, n = 4) after exposure to 1,000 µM benzene and 1,000 µM.

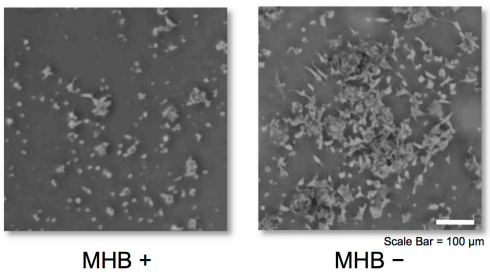

Figure 1. Phase-contrast microscopic observation of mESCs. mESCs were cultured at 37°C in a humidified CO_2 incubator with or without 2% methyl p-hydroxybenzoate (MHB) for 24 h. MHB + or MHB – indicates that mESCs were cultured with or without MHB in the incubator water bath, respectively.

3.3. *Gene ontology term classification of mRNAs*

A two-sample t-test between MHB + and MHB – in the control conditions was conducted, and the RNAs having the 10 lowest p-values were extracted. We then categorized the RNAs according to their Gene Ontology

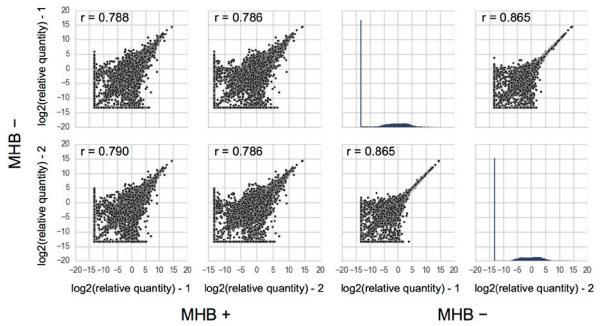

Figure 2. Scatter plot for gene expression pattern determined by RNA-seq under DMSO (control) conditions. mESCs were cultured at 37°C in a humidified CO_2 incubator with DMSO and with or without 2% MHB for 24 h. MHB + or MHB – indicates that mESCs were cultured with or without MHB in the incubator water bath, respectively. The relative quantitative values (FPKMs from RNA-seq data) of 32,586 RNAs were plotted on MHB + versus MHB – (n = 2; left four panels), and between replicated samples of MHB – (n = 2; right four panels), respectively. MHB + and MHB – experiments were performed in duplicate, and each "r" indicates correlation coefficient. Same experiments (MHB – "-1 versus -1" or "-2 versus -2"; right two panels) also indicates that histogram of relative quantitative values.

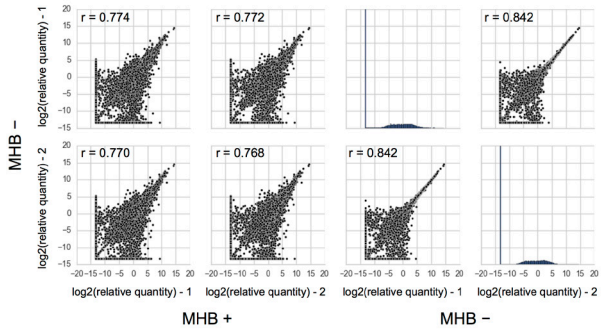

Figure 3. Scatter plot for gene expression pattern determined by RNA-seq under benzene conditions. mESCs were cultured at 37°C in a humidified CO_2 incubator with 1,000 µM benzene and with or without 2% MHB for 24 h. MHB + or MHB – indicates that mESCs were cultured with or without MHB in the incubator water bath, respectively. The relative quantitative values (FPKMs from RNA-seq data) of 32,586 RNAs were plotted on MHB – versus MHB + (n = 2; left four panels), and between replicated samples of MHB – (n = 2; right four panels), respectively. MHB + and MHB – experiments were performed in duplicate, and each "r" indicates correlation coefficient. Same experiments (MHB – "-1 versus -1" or "-2 versus -2"; right two panels) also indicates that histogram of relative quantitative values.

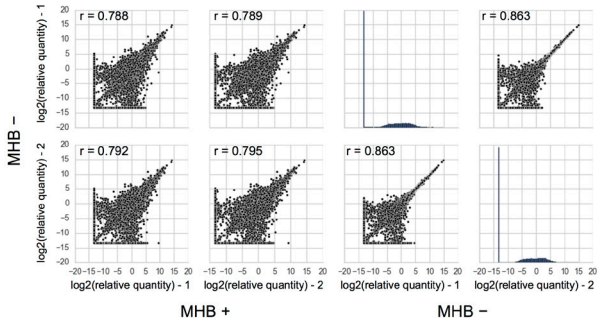

Figure 4. Scatter plot for gene expression pattern determined by RNA-seq under chloroform conditions. mESCs were cultured at 37°C in a humidified CO_2 incubator with 1,000 μM chloroform and with or without 2% MHB for 24 h. MHB + or MHB – indicates that mESCs were cultured with or without MHB in the incubator water bath, respectively. The relative quantitative values (FPKMs from RNA-seq data) of 32,586 RNAs were plotted on MHB – versus MHB + (n = 2; left four panels), and between replicated samples of MHB – (n = 2; right four panels), respectively. MHB + and MHB – experiments were performed in duplicate, and each "r" indicates correlation coefficient. Same experiments (MHB – "-1 versus -1" or "-2 versus -2"; right two panels) also indicates that histogram of relative quantitative values.

(GO) term. We observed significant differences for the GO terms classified as poly(A) RNA binding, chromatin binding, and RNA splicing between MHB + and MHB –. Moreover, cell maturation was also present in the list of GO terms (Table 1). These results suggest that MHB affects not only basic molecular processes but also cell growth. We also conducted the same analysis for the chemical-treated conditions. For the benzene-treated conditions, we observed significant differences for the GO terms classified as cell cycle, RNA splicing, and cell proliferation between MHB + and MHB – (Table 2). On the other hand, for the chloroform-treated conditions, we observed significant differences for the GO terms classified as cell cycle, positive regulation of cell migration, and programmed necrotic cell death between MHB + and MHB – (Table 3). We also examined the FPKM values of cell growth-related genes between duplicated samples of MHB –. We focused on the genes in Tables 1-3 [tumor suppressor candidate 2 (*Tusc2*), centrosomal protein 131(*Cep131*), RB transcriptional corepressor like 1 (*Rbl1*), transcription factor Dp-1

Table 1. Functional classification of RNAs having the 10 lowest P-values under DMSO (control) conditions

Refseq	Gene name	MHB +/MHB –	*p*-value	GO term	Definition
NM_025669	*Pnisr*	111954	7.29×10^{-10}	0044822	poly(A) RNA binding
NM_025891	*Smarcd3*	26184	8.08×10^{-10}	0003682	chromatin binding
NM_173441	*Iws1*	81056	1.53×10^{-9}	0008380	RNA splicing
NM_008717	*Zfp638*	141698	2.39×10^{-9}	0003677	DNA binding
NM_053182	*Pag1*	6244	4.60×10^{-9}	0042169	SH2 domain binding
NR_073524	*Slfn10-ps*	4809	3.02×10^{-8}	-	-
NM_001204333	*Cyp4f14*	1060	5.40×10^{-8}	0050544	arachidonic acid binding
NM_172728	*Creb5*	3700	7.91×10^{-8}	0003677	DNA binding
NM_001024922	*Ddx49*	344879	1.06×10^{-7}	0005524	ATP binding
NM_019742	*Tusc2*	27527	1.34×10^{-7}	0048469	cell maturation

Table 2. Functional classification of RNAs having the 10 lowest P-values under benzene conditions

Refseq	Gene name	MHB +/MHB –	*p*-value	GO term	Definition
NM_009734	*Cep131*	20390	1.58×10^{-9}	0007049	cell cycle
NM_001162922	*Zfp931*	7742	3.37×10^{-9}	0008150	biological process
NM_011249	*Rbl1*	27763	4.71×10^{-9}	0007049	cell cycle
NM_008717	*Zfp638*	58978	7.96×10^{-9}	0008380	RNA splicing
NR_037683	*Snord42b*	74957	1.07×10^{-8}	-	-
NM_001164769	*Fbxw2*	0.000032	2.58×10^{-8}	0005515	protein binding
NM_007460	*Ap3d1*	0.000054	3.91×10^{-8}	0072657	protein localization to membrane
NM_010832	*Msl3*	3004	4.21×10^{-8}	0006325	chromatin organization
NM_011661	*Tyr*	43861	4.90×10^{-8}	0008283	cell proliferation
NM_001040072	*Nynrin*	0.000009	5.27×10^{-8}	0003676	nucleic acid binding

Table 3. Functional classification of RNAs having the 10 lowest P-values under chloroform conditions

Refseq	Gene name	MHB +/MHB –	*p*-value	GO term	Definition
NM_001291768	*Tfdp1*	80940	1.69×10^{-7}	0007049	cell cycle
NM_134087	*Fam83h*	44282	1.84×10^{-7}	0030335	positive regulation of cell migration
NM_001142697	*Tpgs2*	38260	1.86×10^{-7}	0005575	cellular_component
NM_027777	*Pex1*	11049	2.06×10^{-7}	0005524	ATP binding
NM_001004142	*Nlrp1a*	72514	2.21×10^{-7}	0097300	programmed necrotic cell death
NM_001033378	*A430078G23Rik*	8986	2.31×10^{-7}	-	-
NM_028815	*Cep97*	7395	2.55×10^{-7}	0030030	cell projection organization
NM_001162973	*Lrrc51*	4377	2.58×10^{-7}	0005575	cellular_component
NM_133907	*Ube3c*	177872	2.67×10^{-7}	0004842	ubiquitin-protein transferase activity
NM_001004066	*Zfp386*	16820	3.12×10^{-7}	0006355	regulation of transcription, DNA-templated

(*Tfdp1*), and tyrosinase (*Tyr*)]. The FPKM values of cell maturation-related gene, *Tusc2*, were 3.7 and 3.8 in duplicate. Those of cell cycle-related genes, *Cep131*, *Rbl1*, and *Tfdp1*, were 0.55 and 0.53, 3.1 and 3.0, and 25.9 and 29.0, respectively. Those of cell proliferation-related gene, Tyr, were 7.8 and 8.4. These results indicate that cell growth-related genes were not significantly different between duplicated samples of MHB –.

4. Discussion

Our results show that MHB has a growth inhibitory effect on cultured mESCs and that the use of MHB also affects gene expression patterns both in control conditions and in chemical-treated conditions. In out experiments, MHB was not directly added into the culture medium; therefore, we considered that aerosol of MHB from the water bath of incubator affected those above-mentioned effects to cultured cells. The GO term analyses indicate that MHB clearly affects cell growth pathways in both control conditions and chemical-treated conditions. Moreover, the effect of MHB cannot be accounted for by subtracting the gene expression pattern under control conditions from those under chemical-treated conditions. This is because the GO terms that appeared under the control conditions (Table 1) are not identical to those under the chemical-treated conditions (Tables 2 and 3). Therefore, the simple setting of control conditions in cell-based assays is not adequate.

This study is the first to demonstrate the inconvenient effect of MHB on cultured mammalian cells used for gene expression analyses in cell-based toxicity assays. This effect has, to date, been neglected. We suggest that other chemicals commonly used as incubator water bath preservatives should be reconsidered in terms of their biological effects on cultured cells.

Acknowledgements

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