

Deregulations of DNA damage-responsive genes, genes involved in the endocrine system, in an advanced in vitro 3D zebrafish hepatic cell model after exposure to Bisphenol A (BPA) and its emerging alternatives BPAF, BPAP and BPPH.

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Bisphenol AF (BPAF), Bisphenol AP (BPAP), and Bisphenol PH (BPPH) are being introduced into consumer products to replace BPA and are subsequently detected in ecosystems. This study investigates the genotoxic and endocrine-disruptive potential of these emerging bisphenols using a 3D *in vitro* liver spheroid model derived from *Danio rerio* (ZFL cell line), on the transcriptional level. The selected genes targeted DNA damage response pathways (TP53, NER, BER) and endocrine-related signalling (Table 1).

ZFL spheroids were prepared by a force floating method as described by Štampar et al. (2019)¹. Four-day-old ZFL spheroids were exposed to BPA (50 and 200 µM), BPAF (25 and 100 µM), BPAP (25 and 100 µM), and BPPH (10 and 50 µM) for 24 (Table 2) and 96 (Table 3) hours. Following exposure, total RNA was extracted using the RNeasy® Mini Kit (Qiagen, Germany). RNA quality and quantity were assessed prior to reverse transcription (Applied Biosystems, USA), followed by gene-specific preamplification (TATAA PreAmp GrandMasterMix, Tataa Biocenter, Sweden). Gene expression analysis was performed using TaqMan Gene Expression Assays (Applied Biosystems, USA) on the Fluidigm One 48.48 Dynamic Array IFC microfluidic system as described by Štern et al. (2024)².

The generated data was analysed using the Fluidigm Gene Expression Analysis Software and with a free-accessible web program, quantGenious³. The difference in gene expression greater than 1.5-fold was considered a biologically important up/downregulation (relative expression >1.5 or <0.66, respectively). Statistically significant differences were analysed using ANOVA and Dunnett's multiple comparison test in GraphPad Prism v9 (GraphPad Software, San Diego, CA, USA).

Table 1: Taqman Gene Expression Assays used for the toxicogenomic analysis.

| Assay ID | Gene abbr. | Gene name | Gene function group |
|-------------------------------|-----------------|---|---------------------|
| Dr03432748_m1 | <i>eef1a1l1</i> | eukaryotic translation elongation factor 1 alpha 1, like 1 | housekeeping |
| Dr03094395_m1 | <i>rlp8</i> | ribosomal protein L8 | housekeeping |
| Dr03432610_m1 | <i>actb</i> | actin, beta 1 | housekeeping |
| Dr03112089_m1 | <i>tp53</i> | tumor protein p53 | DNA damage response |
| Dr03425943_m1 | <i>gadd45a</i> | growth arrest and DNA-damage-inducible, alpha | DNA damage response |
| Dr03074096_m1 | <i>mdm2</i> | MDM2 proto-oncogene | DNA damage response |
| Dr03122037_m1 | <i>xrcc5</i> | X-ray repair complementing defective repair in Chinese hamster cells 5 | DNA damage response |
| Dr03120437_m1 | <i>ercc3</i> | excision repair cross-complementing rodent repair deficiency, complementation group 3 | DNA damage response |
| Dr03169669_s1 | <i>ogg1</i> | 8-oxoguanine DNA glycosylase | DNA damage response |
| Dr03103722_m1 | <i>casp2</i> | caspase 2, apoptosis-related cysteine protease | Apoptosis |
| Dr03432977_m1 | <i>baxa</i> | bcl2-associated X protein, a | Apoptosis |
| Dr03091049_m1 | <i>bcl2</i> | B-cell leukemia/lymphoma 2 | Apoptosis |
| Dr03131880_m1 | <i>gnrh2</i> | gonadotropin-releasing hormone 2 | Endocrine system |
| Dr03160560_s1 | <i>ar</i> | androgen receptor | Endocrine system |
| Dr03093579_m1 | <i>esr1</i> | estrogen receptor 1 | Endocrine system |
| Dr03074408_m1 | <i>esr2a</i> | estrogen receptor a | Endocrine system |
| Dr03150586_m1 | <i>esr2b</i> | estrogen receptor b | Endocrine system |
| Dr03191564_sH | <i>vtg4</i> | vitellogenin 4 | Vitellogenesis |

Table 2: The fold change of the expression of selected genes involved in DNA damage response and hormone regulation after 24 h of exposure to the tested BPs.

| Gene | Sample | SC | BPA 50 µM | BPA 200 µM | BPAF 25 µM | BPAF 100 µM | BPAP 25 µM | BPAP 100 µM | BPPH 10 µM | BPPH 50 µM | PC |
|----------------|--------|--------|--------------|-------------------|---------------|----------------|---------------|----------------|---------------|---------------|-------------------|
| <i>tp53</i> | Mean | 1.00 | 1.30 | 2.18 | 1.53 | 1.79 | 1.17 | 1.44 | 0.92 | 1.20 | 1.01 |
| | SD | 0.24 | 0.25 | 1.15 | 0.37 | 0.77 | 0.24 | 0.07 | 0.19 | 0.37 | 0.35 |
| | p | 1.0000 | 0.6869 | 0.1077 | 0.4704 | 0.2804 | 0.8139 | 0.5525 | 0.9088 | 0.7856 | 0.9946 |
| <i>gadd45a</i> | Mean | 1.00 | 1.26 | 2.45 | 1.87 | 2.08 | 1.19 | 1.64 | 1.00 | 1.25 | 4.76 |
| | SD | 0.53 | 0.60 | 0.72 | 0.50 | 0.56 | 0.27 | 0.31 | 0.41 | 0.57 | 2.79 |
| | p | 1.0000 | 0.7279 | 0.0483 | 0.2340 | 0.1394 | 0.7937 | 0.3823 | 0.9964 | 0.7343 | <0,0001 |
| <i>mdm2</i> | Mean | 1.00 | 1.61 | 2.50 | 1.03 | 2.45 | 1.26 | 0.60 | 1.11 | 3.65 | 0.95 |
| | SD | 0.43 | 0.64 | 1.75 | 0.29 | 1.36 | 0.07 | 0.46 | 0.64 | 4.19 | 0.45 |
| | p | 1.0000 | 0.4058 | 0.0407 | 0.9670 | 0.0490 | 0.7257 | 0.5825 | 0.8767 | 0.0003 | 0.9453 |
| <i>xrcc5</i> | Mean | 1.00 | 0.96 | 0.64 | 1.29 | 1.17 | 0.86 | 1.12 | 0.84 | 0.92 | 2.86 |
| | SD | 0.23 | 0.13 | 0.15 | 0.32 | 0.47 | 0.12 | 0.20 | 0.10 | 0.21 | 2.12 |
| | p | 1.0000 | 0.9578 | 0.6233 | 0.6893 | 0.8162 | 0.8481 | 0.8741 | 0.8232 | 0.9084 | 0.0240 |
| <i>ercc3</i> | Mean | 1.00 | 1.66 | 1.54 | 2.30 | 3.33 | 1.81 | 1.47 | 1.45 | 1.41 | 2.16 |
| | SD | 0.27 | 0.35 | 0.47 | 0.95 | 1.72 | 0.22 | 0.23 | 0.41 | 0.18 | 0.74 |
| | p | 1.0000 | 0.3675 | 0.4644 | 0.0770 | 0.0046 | 0.2690 | 0.5227 | 0.5392 | 0.5779 | 0.1591 |
| <i>ogg1</i> | Mean | 1.00 | 0.97 | 1.80 | 1.36 | 2.52 | 0.96 | 1.95 | 0.83 | 0.85 | 0.93 |
| | SD | 0.54 | 0.45 | 0.61 | 0.59 | 1.82 | 0.23 | 0.58 | 0.40 | 0.11 | 0.66 |
| | p | 1.0000 | 0.9638 | 0.3279 | 0.6254 | 0.0383 | 0.9538 | 0.1960 | 0.8140 | 0.8532 | 0.9324 |
| <i>casp2</i> | Mean | 1.00 | 1.05 | 0.81 | 1.38 | 1.72 | 1.07 | 1.13 | 1.15 | 0.99 | 1.41 |
| | SD | 0.12 | 0.03 | 0.08 | 0.39 | 1.49 | 0.21 | 0.24 | 0.16 | 0.12 | 0.32 |
| | p | 1.0000 | 0.9428 | 0.7931 | 0.6060 | 0.3273 | 0.9261 | 0.8622 | 0.8344 | 0.9892 | 0.6191 |
| <i>baxa</i> | Mean | 1.00 | 1.06 | 1.77 | 1.31 | 1.28 | 1.07 | 1.20 | 0.96 | 1.16 | 2.39 |
| | SD | 0.09 | 0.03 | 0.33 | 0.26 | 0.32 | 0.06 | 0.06 | 0.15 | 0.15 | 1.62 |
| | p | 1.0000 | 0.9400 | 0.2968 | 0.6747 | 0.6989 | 0.9204 | 0.7885 | 0.9550 | 0.8277 | 0.0587 |
| <i>bcl2</i> | Mean | 1.00 | 0.91 | 0.46 | 1.12 | 1.04 | 0.89 | 1.46 | 0.94 | 1.22 | 1.94 |
| | SD | 0.34 | 0.17 | 0.07 | 0.28 | 0.56 | 0.24 | 0.40 | 0.27 | 0.63 | 1.30 |
| | p | 1.0000 | 0.9071 | 0.4640 | 0.8670 | 0.9552 | 0.8781 | 0.5273 | 0.9393 | 0.7642 | 0.1984 |
| <i>gnrh2</i> | Mean | 1.00 | 0.89 | 1.44 | 0.20 | 0.55 | 1.17 | 1.43 | 0.94 | 1.38 | 0.46 |
| | SD | 0.30 | 0.48 | 1.29 | 0.12 | 0.58 | 0.69 | 0.54 | 0.64 | 0.46 | 0.56 |
| | p | 1.0000 | 0.8764 | 0.5504 | 0.2744 | 0.5423 | 0.8159 | 0.5615 | 0.9336 | 0.6032 | 0.4653 |
| <i>ar</i> | Mean | 1.00 | 1.05 | 4.67 | 1.73 | 2.51 | 1.09 | 1.79 | 0.80 | 1.27 | 5.58 |
| | SD | 0.48 | 0.42 | 4.14 | 0.59 | 1.63 | 0.22 | 0.20 | 0.15 | 0.41 | 4.79 |
| | p | 1.0000 | 0.9407 | <0,0001 | 0.3224 | 0.0401 | 0.9032 | 0.2817 | 0.7814 | 0.7141 | <0,0001 |
| <i>esra</i> | Mean | 1.00 | 0.65 | 0.37 | 1.32 | 1.31 | 0.64 | 2.56 | 1.22 | 2.28 | 0.20 |
| | SD | 0.63 | 0.14 | 0.38 | 0.68 | 0.84 | 0.13 | 0.65 | 0.56 | 1.59 | 0.28 |
| | p | 1.0000 | 0.6378 | 0.3921 | 0.6582 | 0.7083 | 0.6196 | 0.0570 | 0.7679 | 0.1197 | 0.3305 |
| <i>esrb</i> | Mean | 1.00 | 0.88 | 1.04 | 0.83 | 1.23 | 1.74 | 1.47 | 1.14 | 1.91 | 1.20 |
| | SD | 0.09 | 0.12 | 0.24 | 0.17 | 0.47 | 0.27 | 0.41 | 0.08 | 0.57 | 0.41 |
| | p | 1.0000 | 0.8639 | 0.9581 | 0.8125 | 0.7472 | 0.3089 | 0.5135 | 0.8474 | 0.209 | 0.781 |
| <i>vtgr4</i> | Mean | 1.00 | 0.79 | 3.27 | 1.13 | 1.81 | 0.92 | 1.69 | 0.68 | 1.23 | 3.02 |
| | SD | 0.64 | 0.41 | 3.13 | 0.61 | 1.63 | 0.18 | 0.45 | 0.34 | 0.89 | 2.09 |
| | P | 1.0000 | 0.7782 | 0.0021 | 0.8561 | 0.2687 | 0.9095 | 0.3461 | 0.6596 | 0.7532 | 0.0143 |

Table 3: The fold change of the expression of selected genes involved in DNA damage response and hormone regulation after 96 h of exposure to the tested BPs.

| Gene | Sample | SC | BPA 50 µM | BPA 200 µM | BPAF 25 µM | BPAF 100 µM | BPAP 25 µM | BPAP 100 µM | BPPH 10 µM | BPPH 50 µM | PC |
|----------------|--------|--------|---------------|---------------|---------------|----------------|---------------|----------------|---------------|---------------|-------------------|
| <i>tp53</i> | Mean | 1.00 | 1.04 | 0.59 | 0.87 | 0.97 | 0.83 | 0.73 | 0.87 | 0.86 | 0.49 |
| | SD | 0.13 | 0.03 | 0.08 | 0.17 | 0.12 | 0.09 | 0.12 | 0.04 | 0.18 | 0.11 |
| | p | 1.0000 | 0.9169 | 0.2299 | 0.7082 | 0.9231 | 0.6224 | 0.4321 | 0.7047 | 0.6858 | 0.1309 |
| <i>gadd45a</i> | Mean | 1.00 | 1.25 | 1.88 | 1.08 | 1.07 | 1.14 | 0.86 | 0.79 | 0.94 | 3.07 |
| | SD | 0.12 | 0.08 | 0.75 | 0.25 | 0.22 | 0.27 | 0.23 | 0.06 | 0.27 | 1.22 |
| | p | 1.0000 | 0.4525 | 0.0097 | 0.8074 | 0.8398 | 0.6828 | 0.6754 | 0.5350 | 0.8681 | <0.0001 |
| <i>mdm2</i> | Mean | 1.00 | 1.12 | 0.63 | 0.76 | 0.95 | 1.14 | 1.22 | 0.98 | 0.72 | 0.39 |
| | SD | 0.24 | 0.16 | 0.18 | 0.24 | 0.20 | 0.85 | 0.50 | 0.26 | 0.12 | 0.03 |
| | p | 1.0000 | 0.7207 | 0.2718 | 0.4807 | 0.8714 | 0.6848 | 0.5254 | 0.9485 | 0.4087 | 0.1071 |
| <i>xrcc5</i> | Mean | 1.00 | 1.02 | 1.25 | 1.10 | 1.18 | 1.13 | 0.80 | 0.98 | 1.24 | 1.50 |
| | SD | 0.11 | 0.20 | 0.29 | 0.05 | 0.23 | 0.27 | 0.40 | 0.05 | 0.22 | 0.09 |
| | p | 1.0000 | 0.9446 | 0.4534 | 0.7746 | 0.5907 | 0.7040 | 0.5561 | 0.9537 | 0.4787 | 0.1444 |
| <i>ercc3</i> | Mean | 1.00 | 0.93 | 1.23 | 1.10 | 1.10 | 0.95 | 0.97 | 1.04 | 1.13 | 1.26 |
| | SD | 0.10 | 0.03 | 0.16 | 0.20 | 0.20 | 0.09 | 0.36 | 0.23 | 0.16 | 0.17 |
| | p | 1.0000 | 0.8323 | 0.5035 | 0.7614 | 0.7771 | 0.8795 | 0.9249 | 0.9066 | 0.7083 | 0.4864 |
| <i>ogg1</i> | Mean | 1.00 | 1.03 | 0.84 | 0.94 | 0.96 | 1.14 | 1.02 | 0.71 | 1.42 | 2.42 |
| | SD | 0.24 | 0.58 | 0.79 | 0.57 | 0.73 | 0.12 | 0.40 | 0.33 | 1.20 | 0.77 |
| | p | 1.0000 | 0.9264 | 0.6343 | 0.8630 | 0.9105 | 0.6792 | 0.9571 | 0.3882 | 0.2180 | 0.0002 |
| <i>casp2</i> | Mean | 1.00 | 0.99 | 1.04 | 1.12 | 1.03 | 1.23 | 1.19 | 1.09 | 1.05 | 1.38 |
| | SD | 0.13 | 0.10 | 0.13 | 0.01 | 0.21 | 0.32 | 0.14 | 0.19 | 0.13 | 0.01 |
| | p | 1.0000 | 0.9729 | 0.9090 | 0.7320 | 0.9277 | 0.4917 | 0.5701 | 0.7893 | 0.8943 | 0.3109 |
| <i>baxa</i> | Mean | 1.00 | 1.07 | 1.11 | 0.94 | 0.95 | 1.11 | 1.23 | 1.01 | 1.00 | 1.19 |
| | SD | 0.16 | 0.16 | 0.10 | 0.19 | 0.19 | 0.48 | 0.31 | 0.24 | 0.05 | 0.44 |
| | p | 1.0000 | 0.8403 | 0.7351 | 0.8668 | 0.8932 | 0.7449 | 0.5059 | 0.9704 | 0.9995 | 0.5817 |
| <i>bcl2</i> | Mean | 1.00 | 1.28 | 1.49 | 1.31 | 1.23 | 1.23 | 1.19 | 1.09 | 1.45 | 2.13 |
| | SD | 0.06 | 0.23 | 0.30 | 0.06 | 0.45 | 0.33 | 0.40 | 0.21 | 0.46 | 0.55 |
| | p | 1.0000 | 0.4153 | 0.1518 | 0.3618 | 0.4955 | 0.5065 | 0.5661 | 0.7995 | 0.1895 | 0.0010 |
| <i>gnrh2</i> | Mean | 1.00 | 1.96 | 2.06 | 1.30 | 1.28 | 2.30 | 0.98 | 1.23 | 1.68 | 4.79 |
| | SD | 0.18 | 0.53 | 0.55 | 0.44 | 0.83 | 1.55 | 0.59 | 0.11 | 0.27 | 1.86 |
| | p | 1.0000 | 0.0050 | 0.0018 | 0.3812 | 0.4068 | 0.0001 | 0.9548 | 0.5046 | 0.0470 | <0.0001 |
| <i>ar</i> | Mean | 1.00 | 1.00 | 0.70 | 0.87 | 0.92 | 1.01 | 0.89 | 0.63 | 1.10 | 2.33 |
| | SD | 0.29 | 0.54 | 0.64 | 0.49 | 0.64 | 0.17 | 0.31 | 0.31 | 0.72 | 1.19 |
| | p | 1.0000 | 0.9946 | 0.3714 | 0.7104 | 0.8188 | 0.9802 | 0.7550 | 0.2696 | 0.7738 | 0.0005 |
| <i>esra</i> | Mean | 1.00 | 0.90 | 0.56 | 1.61 | 0.70 | 2.15 | 1.27 | 1.03 | 1.60 | 1.27 |
| | SD | 0.29 | 0.02 | 0.00 | 1.12 | 0.04 | 0.91 | 0.13 | 0.36 | 0.28 | 0.35 |
| | p | 1.0000 | 0.8015 | 0.2496 | 0.0730 | 0.4324 | 0.0008 | 0.4259 | 0.9327 | 0.0768 | 0.4781 |
| <i>esrb</i> | Mean | 1.00 | 0.90 | 0.56 | 1.61 | 0.70 | 2.15 | 1.27 | 1.03 | 1.60 | 1.27 |
| | SD | 0.29 | 0.02 | 0.00 | 1.12 | 0.04 | 0.91 | 0.13 | 0.36 | 0.28 | 0.35 |
| | p | 1.0000 | 0.7193 | 0.9123 | 0.619 | 0.4992 | 0.0333 | 0.1712 | 0.6867 | 0.0087 | 0.5601 |
| <i>vtgr4</i> | Mean | 1.00 | 0.94 | 0.79 | 0.72 | 0.75 | 0.98 | 0.90 | 0.61 | 1.17 | 1.96 |
| | SD | 0.26 | 0.55 | 0.86 | 0.27 | 0.41 | 0.39 | 0.44 | 0.31 | 0.88 | 0.97 |
| | p | 1.0000 | 0.8693 | 0.5401 | 0.4143 | 0.4630 | 0.9486 | 0.7772 | 0.2505 | 0.6146 | 0.0049 |

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