

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>vtg4</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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