ANEUPLOIDY AND TOXICITY ASSESSMENT OF BISPHENOL A, F, AND S

By

Aaron Zhunming Lai

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APPROVED

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Dr. David Eastmond
Department of Cell Biology & Neuroscience

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Dr. Richard Cardullo, Howard H Hays Jr. Chair and Faculty Director, University Honors
Interim Vice Provost, Undergraduate Education

Abstract

Used widely in producing plastics, packaging, and other coatings, Bisphenol A (BPA) is a chemical to which consumers are commonly exposed. To avoid customer exposure, some companies have replaced BPA with Bisphenol F (BPF) and Bisphenol S (BPS). The goal of this study was to determine if BPF and BPS exhibit similar toxic characteristics to BPA.

Using the lymphoblastoid cell line TK6, BPA was the most toxic, followed by BPF and then BPS. Also, BPA showed elevated levels of aneuploidy at doses as low as 250 μM, while BPF and BPS showed generally similar levels of aneuploidy at higher, more toxic doses. Since BPF and BPS did show aneuploidy at higher doses, this raises questions about whether the aneuploidy was caused by toxicity or involved a specific mechanism, so further experiments on more sensitive cell lines are warranted.

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Introduction

Bisphenol A (BPA) is a common component in consumer items, such as food packaging, receipts, and plastic polycarbonate bottles, and it has been used for some time. However, BPA's harmful effects have been studied intensively, and some agencies, such as the FDA, have taken actions to limit its usage.

In place of BPA, some companies have moved towards using Bisphenol F and Bisphenol S, which chemically resemble BPA. These chemicals have not been investigated as thoroughly, and some papers suggest that they have similar effects, such as BPF possibly being moderately cytotoxic and genotoxic (Audebart et al. 2011). Using the TK6 lymphoblastoid cell line, Bisphenol A, F, and S's effects will be examined and compared in these experiments.

Materials and Methods

Cell Culture

For these experiments, human lymphoblast cells from the TK6 line from the American Type Culture Collection were used to observe toxicity. Cells were cultivated in RPMI 1640, 2mM L-glutamine, 100 IU penicillin/100 μg/mL streptomycin and 10% heat-inactivated iron –supplemented calf serum. The cells were regularly maintained at a concentration between $2.5x10^5$ to $10x10^5$ cells/mL at 37^oC and in 5% CO_2 in a humidified incubator.

Chemicals

Bisphenol A, Bisphenol F, and Bisphenol S were purchased from Sigma Chemical Company. Colcemid, propidium iodide, RNase A and Tween 20 were all purchased from Sigma Chemical Company (St. Louis, MO). DPBS without Ca^{2+} and Mg^{2+} (Media Tech,

Herndon, VA) was used as buffer and sheath fluid for the flow cytometer. The primary antibody, Phospho-histone H3(Ser 10)6G3 monoclonal antibody, was purchased from Cell Signaling Technologies (Beverly, MA) and the secondary antibody, Alexa Fluor 488-labelled goat anti-mouse antibody, was purchased from Life Technologies (Eugene, OR).

Dose-Response Curve

For all three bisphenols, dose-response curves were initially run to identify an appropriate dosage that would yield a moderate toxicity on which to base the flow cytometry analyses. Doses of each chemical were prepared in DMSO (dimethyl sulfoxide). The cultures were then treated at 100 µl dose/10mL cell culture, and the dosages were determined based on a logarithmic scale. The dosed cells were harvested 24 hours after dosing.

Bisphenol Treatment and Cell Storage

Similar to the dose-response experiments, doses of each chemical were prepared in DMSO, and cell cultures were dosed at 100 µl dose/10mL. After dosing, the cultures were incubated for 24 hours at 37^{o}C in a CO_2 incubator. At 21 hours, cell cultures were dosed with 0.075 µg/ml colcemid, which would suppress microtubule dynamics and halt mitosis at metaphase. This allows for a build-up of cells in metaphase. Cells would be harvested at 24 hours, and cell numbers were determined using a Coulter Counter (Beckman Coulter, Series Z), providing a measure for relative toxicity.

Cell cultures were then promptly centrifuged for 6 minutes at 1000RPM and the resulting supernatant was aspirated. After re-suspension, the cells were fixed with 70% EtOH at -20 ^{o}C and stored at -20 ^{o}C until analysis.

Immuno-Labelling Cells for Flow Cytometry

To allow cells to be detected via flow cytometry, the cells in the EtOH solution were aspirated and rehydrated with 0.1% Tween 20/PBS. They were then incubated at 37^{o}C for 45 minutes with the primary antibody, phopho-histone H3 (Ser 10) 6G3 monoclonal mouse in PBS (1:400 dilution). They were then incubated for another 45 minutes with the Alexa Fluor 488-labeled secondary antibody (1:1000 dilution in PBS), allowing for visualization of metaphase nuclei. Lastly, the cells were counter-stained with a solution of propidium iodide (25 µg/ml)/RNase A (200 µg/ml).

Flow Cytometer Analysis and Configuration

Samples were analyzed via a single-laser flow cytometer (FACSort, BD Sciences, San Jose, CA) at a 488 nm excitation. Instrumentation settings and data acquisition/analysis were configured with CellQuest software v3.3 (BD Biosciences). The fluorescent emission of the FITC-labeled mitotic cells was collected in the Fl-1 channel, and PI-stained DNA fluorescent emission was collected in the FL-2 channel. Since some polyploid and hyperploid TK6 cells were outside the standard rectangular gate, a slanted upper limit was employed, giving a trapezoid shaped gate that included polyploid cells while excluding possible cell clumps or doublets. Data on the hypoploidy, hyperploidy, polyploidy, and relative mitotic index for each concentration were collected once 2000 mitotic events were reached or until excessive toxicity occurred.

Results

Bisphenol A, F, & S Dose Response & Toxicity

To determine the optimum dose for the Bisphenol experiments, a dose-response experiment for each bisphenol was performed. The resultant cell counts and toxicities for each chemical is shown in Table 1:

Table 1:

Cell counts acquired with Coulter Counter (Beckman Coulter, Series Z). Toxicity percentage calculated with reference to the control dosage, 0 µM.

Bisphenol F Dosage	**Cell Count (x10^5)**	**Toxicity (%)**
0 µM	6.89	0
25 µM	6.76	1.93
35 µM	6.69	2.97
55 µM	6.35	7.83
75 µM	5.68	17.54
120 µM	4.82	30.02
Bisphenol S Dosage	**Cell Count (x10^5)**	**Toxicity (%)**
0 µM	9.22	0
25 µM	9.28	0
55 µM	9.16	0.68
120 µM	8.41	8.78
250 µM	6.50	29.50
500 µM	4.92	46.59
Bisphenol A Dosage	**Cell Count (x10^5)**	**Toxicity (%)**
0 µM	7.38	0
25 µM	6.95	5.83
55 µM	6.61	10.47
120 µM	5.98	18.95
250 µM	3.46	53.15
500 µM	2.40	67.50
1000 µM	2.69	63.51

As each chemical displayed a significant but moderate toxicity at different concentrations (BPA at 250 µM, BPS at 500 µM, BPF above 120 µM), the subsequent experiments for flow cytometry were set up around the different midpoints.

Bisphenol Flow Cytometric Analysis of Aneuploidy-Data

The mean results from the flow cytometer assay are shown below in Tables 2 and 32, including the relative mitotic index, hypoploidy, hyperploidy, and polyploidy values. Figure 2 displays the aneuploidy information from Table 3 for each chemical alone, while Figure 3 displays only hypoploidy, hyperploidy, or polyploidy for all 3 chemicals. Figure 1 graphically juxtaposes the mean cell viability and relative mitotic index, whose data is shown in Table 2.

Table 2:

Cell counts from TK6 cell lines were acquired with Coulter Counter (Beckman Coulter, Series Z), then converted to viability percentage, or percentage of population remaining. Relative mitotic index was measured via flow cytometry.

Bisphenol A	**Viability (%)**	**RMI**
0 µM	100	99.99
25 µM	96.29	91.78
55 µM	89.64	94.45
75 µM	87.77	83.30
120 µM	75.24	83.11
250 µM	50.45	41.95
320 µM	48.68	21.74
400 µM	42.01	1.74
500 µM	40.05	2.06
Bisphenol F	**Viability (%)**	**Relative Mitotic Index (RMI)**
0 µM	100	99.99
25 µM	97.04	99.83
55 µM	91.68	N/A
75 µM	86.27	N/A
120 µM	73.70	65.37
250 µM	54.54	57.45
500 µM	36.48	22
Bisphenol S	**Viability (%)**	**RMI**
0 µM	100	100.01
25 µM	98.52	103.05
120 µM	89.12	N/A
250 µM	68.82	50.14
500 µM	59.51	28.41
750 µM	56.77	20.12

Figure 1:

Using data from Table 2, relative mitotic index (RMI) and cell viability are graphed concurrently here for each chemical.

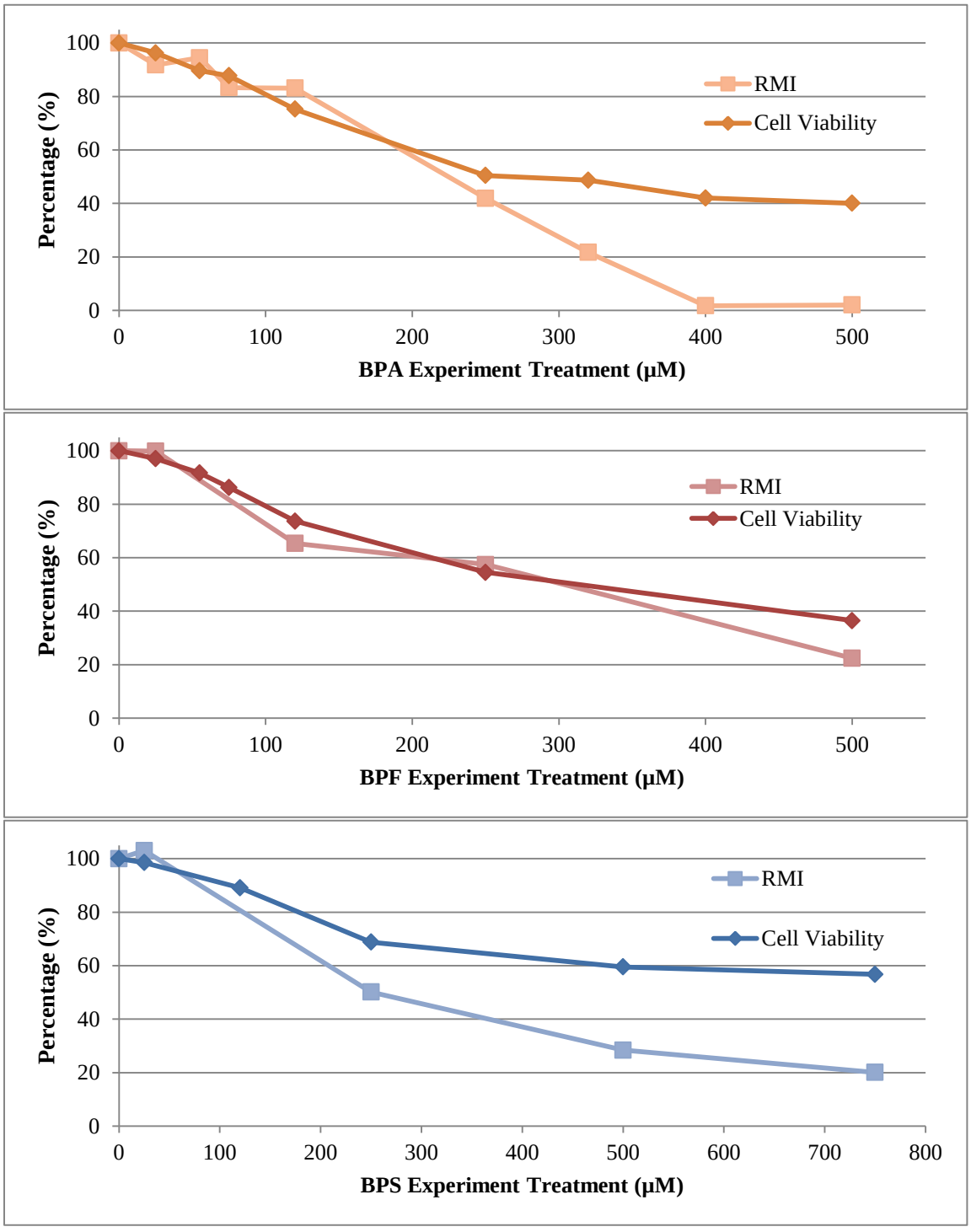


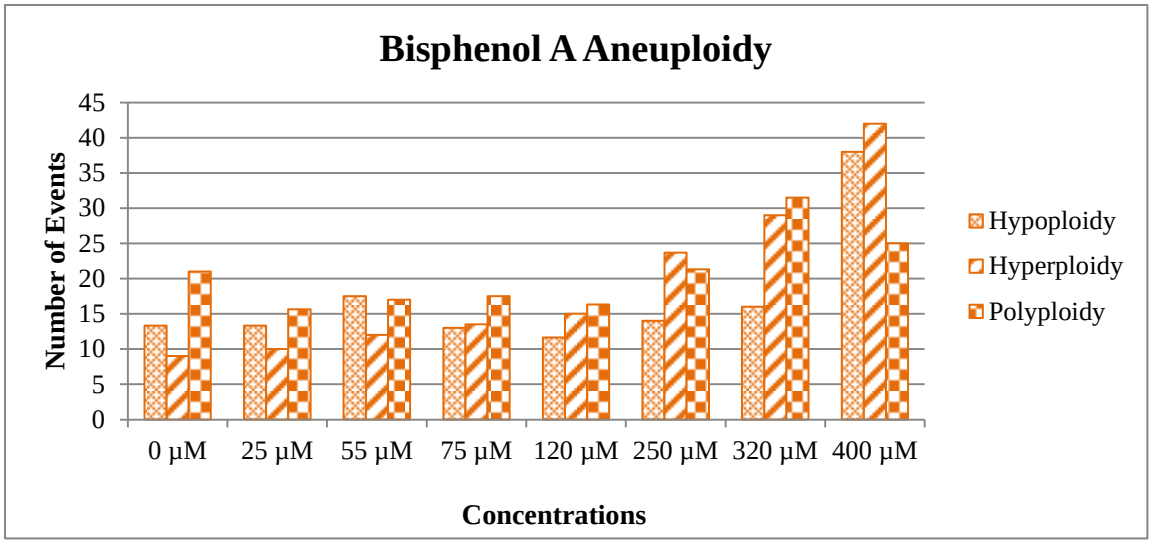
Table 3:

Average relative mitotic index and all aneuploidy events were measured via flow cytometry after TK6 cells were dosed with chemical for 24 hours. The ploidy events are per 2000 cells.

Bisphenol A	**Relative Mitotic Index**	**Hypoploidy (Events)**	**Hyperploidy (Events)**	**Polyploidy (Events)**
0 µM	99.99	13.33	9	21
25 µM	91.78	13.33	10	15.67
55 µM	94.45	17.5	12	17
75 µM	83.30	13	13.5	17.5
120 µM	83.11	11.67	15	16.33
250 µM	41.95	14	23.67	21.33
320 µM	21.74	16	29	31.5
400 µM	1.74	38	42	25
500 µM (toxic)	2.06	0	0	2
Bisphenol F	**Relative Mitotic Index**	**Hypoploidy (Events)**	**Hyperploidy (Events)**	**Polyploidy (Events)**
0 µM	99.99	18.5	13	14.5
25 µM	99.83	18	13.5	18
120 µM	65.37	19	13.5	31.5
250 µM	57.45	18	16	23.5
320 µM, n=1	35	13	20	20
500 µM	22.41	38	132	13
Bisphenol S	**Relative Mitotic Index**	**Hypoploidy (Events)**	**Hyperploidy (Events)**	**Polyploidy (Events)**
0 µM	100.01	13.5	11.5	15
25 µM	103.05	8	14.5	17.5
250 µM	50.14	26	14	26
320 µM, n=1	46	20	14	19
500 µM	28.41	24.5	15.5	20.5
750 µM	20.12	28	70.5	26

Figure 2:

Using data from Table 3, the aneuploidy events for each chemical are shown at their respective concentrations.



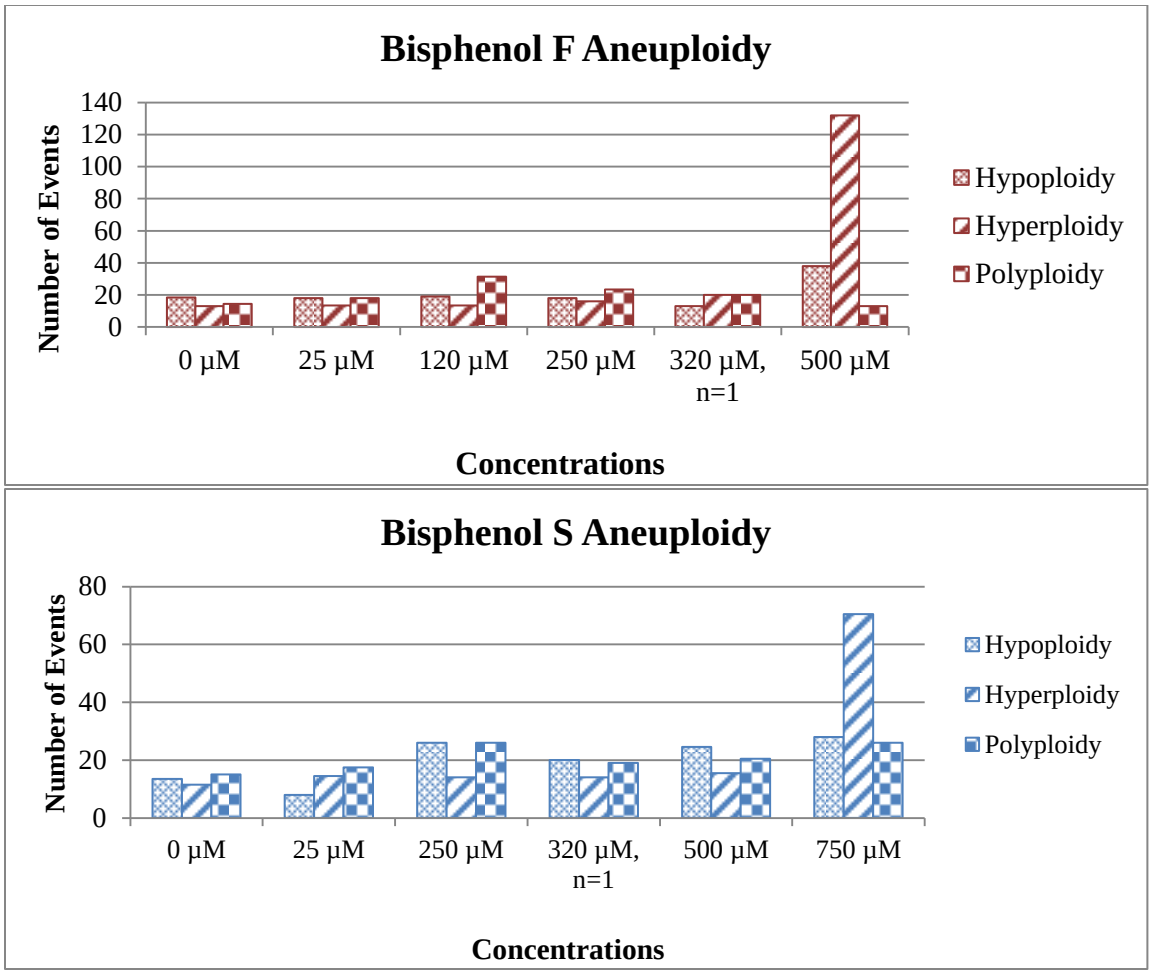
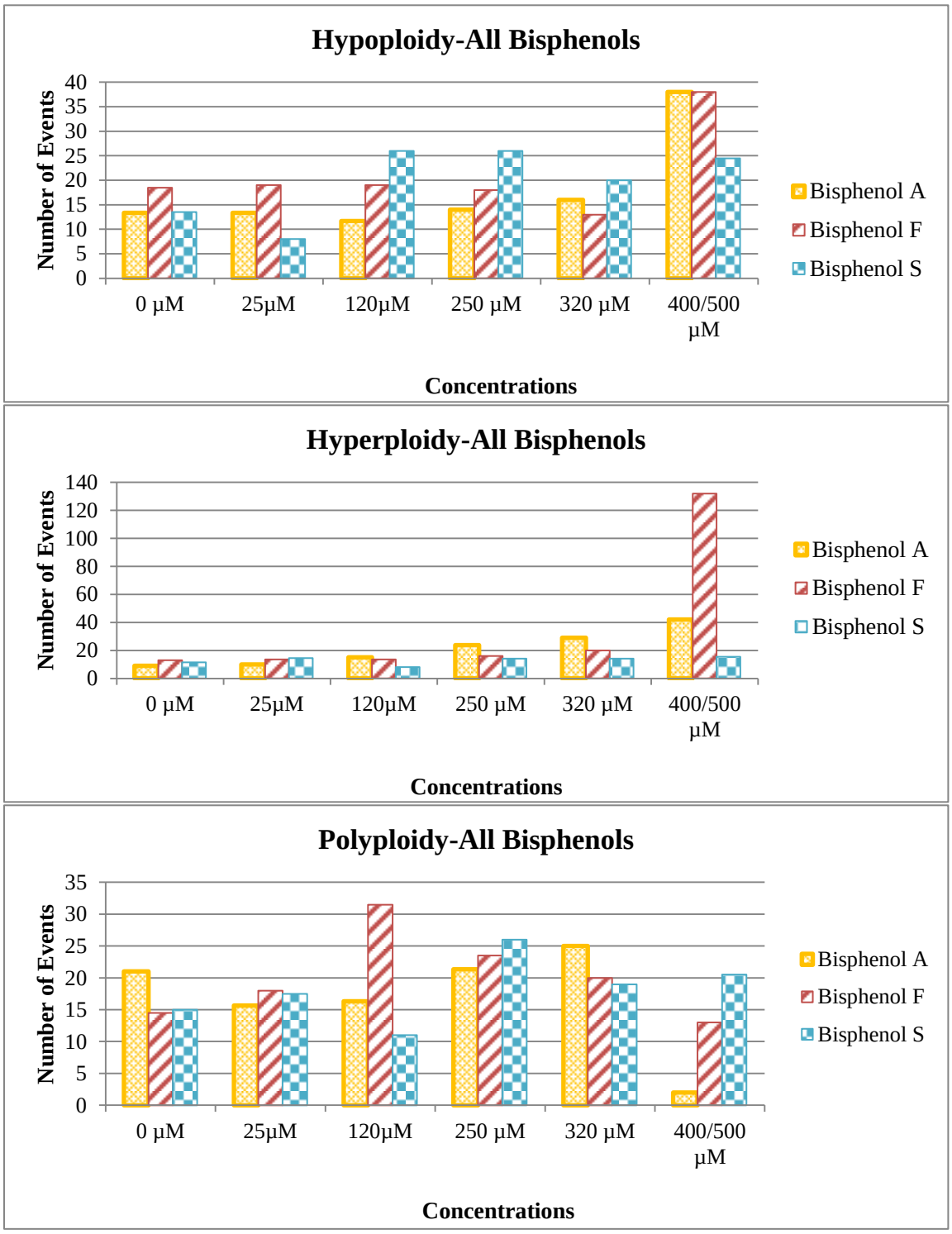


Figure 3:

Taken from Table 3, all aneuploidies are shown on the next page, page 13. For the 400/500µM dose label on the graph, BPA is shown at 400µM and BPF/S are shown at 500µM, as BPA was highly toxic at 500 µM. Results from the 750 µM concentration of BPS are not shown.

Figure 3 (Continued):



As can be seen from Figure 1 and Table 2, Bisphenol A was overall more toxic than its counterparts, as its RMI and cell viability decreased quickly after 250 μM.

The data for each chemical for each type of aneuploidy were analyzed by one-way ANOVA. BPA and BPF did present significant increases in hyperploidy with $p<0.05$, although the increase seen at 500 uM BPF was based on a high value in only one experiment and should be replicated. No differences in aneuploidy events were seen among the 3 chemicals at the lower non-toxic concentrations. However, as toxicity increased, increases in hyperdiploidy were seen for each chemical with BPA being the most potent, followed by BPF. The non-statistically significant increase seen with BPS occurred at the very high 750 uM concentration.

Discussion

This study investigated the cytotoxic and chromosomal effects of bisphenol A and how its currently used relatives might behave similarly or differently, using the TK6 cell line. Bisphenol A became toxic relatively quickly at lower concentrations, and the RMI data reflects this event. However, while BPF and BPS were not as toxic, they were still able to reach over 75% toxicity below 750 μM.

While there was little difference between all three chemicals in aneuploidy at most concentrations, it is interesting to note that cells dosed with BPF and BPS experienced similar amounts to cells dosed with BPA at most concentrations, despite BPA being more toxic overall. Additionally, as the concentrations of BPF and BPA increased, significant increases in hyperploidy were seen in these TK6 cells. As a result, it would be

interesting to observe the bisphenols' effect in vivo, in a living organism. Also, a different or more sensitive cell line may provide more useful data, as it is difficult to distinguish in TK6 cells between aneuploidy caused by toxicity and aneuploidy caused by a chemical-specific mechanism.

As BPA is known to have estrogenic properties (Krisnan, et. al 1993), it would be worth continuing this research on cells with estrogen receptors like the MCF-7 line, now that it has been observed that the other bisphenols have roughly similar effects to BPA. With this, more information on the mode of action for these chemicals could be generated and confirm whether these other bisphenols have similar genotoxic properties.

In summary, in these preliminary experiments, BPA proved to be more toxic than BPF and BPS and appeared to be more potent in inducing aneuploidy as well. Since increases in aneuploidy may be related to the estrogenic properties of these compounds, it would be interesting to conduct follow-up experiments using estrogen-sensitive cells.

Bibliography:

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Krishnan, Aruna V., et al. "Bisphenol-A: an estrogenic substance is released from polycarbonate flasks during autoclaving." Endocrinology 132.6 (1993): 2279-2286.