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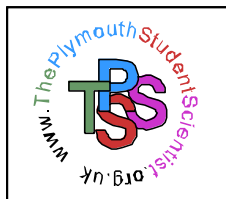
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# The Effects of Bisphenol A on in-vitro Cell Viability of Mammalian Cell Line by Neutral Red Assay

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## Abstract

Bisphenol-A (BPA), a known oestrogenic endocrine disruptor, has many effects in-vivo. However, most studies on its effects concentrate on this oestrogenic property and not on direct cytotoxicity. An in-vitro approach was taken here, showing that BPA has a dose-dependent effect on the viability of Chinese hamster ovary (CHO-K1) cells. Concentrations at which BPA directly generates cytotoxic effects were investigated by exposing CHO-K1 to BPA for 48 hours at 37°C and their viability assessed by their ability to take up and retain the neutral red staining agent. Cells were exposed to tenfold dilutions of BPA from  $1 \times 10^{-4} \text{M}$  to  $1 \times 10^{-9} \text{M}$  and viability measured as a function of absorbance at 540nm wavelength. The greater the absorbance, the more neutral red was successfully taken up by the cells, indicating superior health and general viability. Resulting absorbances ranged from a mean of 2.435A (SEM 0.162) for  $1 \times 10^{-9} \text{M}$  BPA down to 1.924A (SEM 0.103) for  $1 \times 10^{-4} \text{M}$  BPA. A clear relationship between BPA concentration and reduction in absorbance emerged after exposure as assessed by one-way ANOVA. A one-way ANOVA performed on stacked results of  $1 \times 10^{-4} \text{M}$  to  $1 \times 10^{-6} \text{M}$  BPA and stacked results of  $1 \times 10^{-7} \text{M}$  to  $1 \times 10^{-9} \text{M}$  BPA revealed a highly statistically significant difference between these groups ( $p=0.000$ ). This suggests that there is a cytotoxicity "threshold" between  $1 \times 10^{-7} \text{M}$  to  $1 \times 10^{-6} \text{M}$  BPA, over which a proportionately large increase in cytotoxicity is seen, compared to lower concentrations. Analysis of the difference between only the  $1 \times 10^{-7} \text{M}$  to  $1 \times 10^{-6} \text{M}$  by one-way ANOVA fell just short of the 95% confidence interval ( $p=0.058$ ), showing that around these concentrations is where further work need focus to ascertain a highly accurate point of increased cytotoxicity. Whilst this "toxicity threshold" has been elucidated by these analyses, it must be noted that the other concentrations investigated most likely also displayed decreased viability compared to control medium. However, for reasons presented in this paper, the control measurements made were not reliable and so further control testing and comparison will be needed to provide much-needed perspective on the obtained results. The cytotoxic "threshold" of BPA revealed herein further calls for assessment of public health and investigation of exposure to this cytotoxic agent. Recommendations for further studies and the wider implications of the concentrations investigated here are also given in this paper.

**Keywords:** bisphenol A, neutral red, endocrine disruptor, in-vitro, cytotoxicity, public health.

## 1. Introduction

Bisphenol-A (BPA) is a planar molecule that mimics the structure of oestrogen and can activate oestrogen receptors in a manner 1:2000 the potency of normal 17 $\beta$ -estradiol (Bergeron et al., 1999). BPA has also been shown to act on thyroid hormone receptors (Moriyama et al., 2002) (Zoeller et al., 2005) and the monoaminergic nervous system (Negishi et al., 2004), amongst other effects.

BPA is used as a monomer in the manufacture of polycarbonate plastic worldwide and BPA itself is produced in an amount in excess of 6.4 billion pounds per year (vom Saal et al., 2005) and has been shown to leach out of polycarbonate plastic products (Krishnan et al., 1993). Worse, it has been shown that this leaching increases as the product ages. This has been demonstrated using laboratory cages for animals that leached BPA into distilled water (0.3 $\mu$ g BPA/L water) in new cages compared to (310 $\mu$ g/L) in aged cages (a 1033 fold increase), indicating that the longer we are in contact with polycarbonate products and the older those polycarbonate products are, the greater our exposure to BPA (Howdeshell et al., 2003). This is of particular concern to babies fed from polycarbonate bottles or fetuses whose mothers are exposed to BPA as the child will be exposed to BPA in high amounts (the placenta presents no barrier to BPA – (Ikezuki et al., 2002.)) BPA is also increasingly found in our natural environment, leaching from landfill sites and contributing up to 84% of the landfill site's oestrogenicity (Kawagoshi et al., 2003.) This leaching BPA in our environment, aside from other effects mentioned below, can even reduce the fertility of our soil by inhibiting the ability of nitrogen-fixing bacteria to function properly (Fox et al., 2007.)

Many studies have confirmed the oestrogenic and pathogenic effects of BPA in-vivo, mostly in rats and mice, but also in humans. These effects include, but are not limited to, aneugenic effects (Hunt et al., 2003), early onset of puberty (Honma et al., 2002) (Howdeshell et al., 1999), altered reproductive development (Nagel et al., 1997) (Kawai et al., 2003), obesity (Masuno et al., 2002), diabetes (Alonso-Magdalena et al., 2006), altered brain development (Ishido et al., 2004), immune interference (Sawai et al., 2003) (Yoshino et al., 2004), increased cancer cell growth (Vandenberg et al., 2007) (Murray et al., 2007) etc. However, the metabolism of bisphenol-A in a whole-organism setting can distort our understanding of its effects were such metabolism absent. In-vitro studies allow us to see the direct effect of the compound on cells as they are exposed to it in the absence of more complex metabolism for a clear view of its potential effects. Essentially, by using cells in culture, we eliminate the variation the in-vivo studies carry by removing the factor of the hosts' differing abilities to clear an insult of BPA (for example, studies in rats and humans are not comparable due to the presence, in rats, of enterohepatic circulation, greatly altering their processing of BPA). Assessing the toxicological effects of BPA, as opposed to its oestrogenic effects, has not been the main focus of the

literature published on BPA. Cytotoxic effects in addition to oestrogenic effects may well be extremely important in our understanding of the broader range of effects that BPA has on organisms. A mammalian cell line was chosen here to present our results in a manner that is as relevant to humans as possible. This also avoided lengthy ethical approval and would allow the end result to be repeatable without such hassle in a cell line that is well established with very little variation for maximal consistency in results and methods.

The neutral red assay was chosen because it assesses the lysosomal integrity of cells. As the integrity of the lysosome in cells is dependant upon a very wide range of healthy internal machinery, as opposed to a single or a few individual pathways, it provides a good overview of general cellular health as deficient systems inside the cell are likely to impact on the cells ability to retain the neutral red stain. The neutral red assay also results in easily measurable and useable data in the form of absorbances, enabling clear and concise comparison of the effects of agents on the health of the cell. These data can be directly analysed statistically, due to their numeric nature, providing a means of testing for significance as well as more basic comparison. Regarding the choice of cells, a mammalian cell line was chosen to maximise relevance to humans without having to source human cells and the ethical considerations that would involve. CHO-K1 cells have been used for many nutrient and genetic studies in the past and are also useful for viability assays such as this.

BPA has been shown to be metabolised quickly with a half life of around <6 hours (Volkel et al., 2002), after which it is known to be completely metabolised to bisphenol-A glucuronide (Ouchi et al., 2002.) However, BPA has been found at a median level of 3.1ng/ml in plasma of 37 pregnant women (Schönfelder et al., 2002), suggesting a relatively constant exposure at low levels, despite fast metabolism. If indeed, such low concentrations of BPA are being experienced by people as a whole, then it would be prudent to see whether, on a cellular level, such concentrations of BPA caused direct cytotoxic effects. BPA was also found in the urine of 394 tested US adults with a mean result of 1.33 $\mu$ g/L (Calafat et al., 2005.)

More than simply assessing the effect of a low concentration of BPA on mammalian cells in vitro, it was decided to ascertain if there was a particular concentration (or concentrations, in fact) of BPA that showed markedly increased cell cytotoxicity. To investigate a possible dose-dependant relationship, it was decided to use tenfold dilutions from 4x10<sup>-4</sup>M BPA down to extremely low 4x10<sup>-9</sup>M BPA. By plotting the results of all the values inbetween, it was hoped that a clear jump in toxicity would make itself clear.

Alternatively, a spread of concentrations would also elucidate other facts about BPA even if the expected dose-dependant relationship was found to not be the case. For example, if BPA appeared highly toxic at all our tested

concentrations, this would also be a valid result, although testing of concentrations outside those chosen would still be in order. Again, if BPA showed only low toxicity at all concentrations, this should be furthered by testing at other concentrations also to make sure that BPA had a standard cytotoxicity over a wide range of concentrations (although this scenario was deemed to be highly unlikely).

The results of the experiment will provide insight into the effect of BPA on cellular viability in mammalian cells when there is no interference from in-vivo metabolic processes, as previously mentioned. It should also be noted that in-vitro studies do away with differences in health, genetics, age and a host of other factors, allowing a clear cut result to be obtained that is both independent of all these factors but, more importantly, is far more repeatable and, ideally, potentially usable as a basic benchmark for BPA exposure and toxicity in experiments or diagnostics to come.

## 2. Materials and Methods

### 2.1 Cell culture maintenance and passaging

Chinese hamster ovary (CHO-K1) cells were cultured in Ham's F-12 medium supplemented with 10% foetal bovine serum and 2mM glutamine and seeded at a concentration of  $5 \times 10^4$  cells/ml in 8ml of medium in culture flasks. At each passage, three new flasks were seeded at this same concentration. Cells were kept healthy by passage and re-seeding in this manner twice a week (on Tuesdays and Fridays, unless insufficient confluence required a delay.)

Passages involved first selecting which vial of the previous three was not contaminated and showed best confluence to around 90% confluence. This was achieved simply by reverse microscopy and comparison by eye. The method of seeding the other vials from this chosen source involved pouring off medium, washing twice with 5ml PBS (phosphate buffer saline) solution and then trypsinising with 1ml 0.25% trypsin. Trypsin was washed over the surface of the cells simply by tilting the vial for around 20 seconds and then poured off immediately. Cells were then left to detach from the vial and from each other until they had a rounded appearance when viewed under reverse microscopy. Cells were agitated by hand tapping of vial to assist in detaching them from the base of the vial.

New medium was then added (5ml) and the cells resuspended. Cell counts were carried out manually by haemocytometer. From this known concentration of cells per ml, an appropriate volume of cell suspension to provide the correct number of cells to seed a new vial was determined. Medium was added to the new vials first so as to prevent the clumping of cells that would have

occurred if cells were added to a dry vial before medium. Three such vials were prepared and labelled after each passage and then stored at 37°C until the next passage in an incubator.

Example calculation (taken from passage performed on 04/12/2007):

Cells found in each quadrant of haemocytometer = 60, 49, 75, 90

Mean cells per quadrant = 68.5

Cells per  $0.1\text{mm}^3$  = 68.5

Cells per  $1\text{mm}^3$  = 685

Cells per  $1\text{cm}^3$  (cells per 1ml) =  $685000 = 6.85 \times 10^5$  cells

To make up  $4 \times 10^5$  cells total in the vial, this corresponds to  $(4 \times 10^5 / 6.85 \times 10^5) = 0.583\text{ml}$  of our suspension of cells.

Therefore the new vial should be seeded with:

0.583ml cell suspension + 7.417ml medium = 8ml

BPA solution was prepared by dissolving 0.0023g BPA in 50µl DMSO. This was then made up to 100ml by addition of 99.95ml CHO medium. The original concentration of this made up stock solution of BPA in DMSO and medium was  $1 \times 10^{-4}\text{M}$  BPA. This was then made in tenfold dilutions to  $1 \times 10^{-5}\text{M}$  through  $1 \times 10^{-9}\text{M}$  in the following manner:

Concentration (BPA) desired	BPA added (ml)	Medium added (ml)
$1 \times 10^{-4}\text{M}$	5 of stock solution	0
$1 \times 10^{-5}\text{M}$	0.5 of $1 \times 10^{-4}\text{M}$ solution	4.5
$1 \times 10^{-6}\text{M}$	0.5 of $1 \times 10^{-5}\text{M}$ solution	4.5
$1 \times 10^{-7}\text{M}$	0.5 of $1 \times 10^{-6}\text{M}$ solution	4.5
$1 \times 10^{-8}\text{M}$	0.5 of $1 \times 10^{-7}\text{M}$ solution	4.5
$1 \times 10^{-9}\text{M}$	0.5 of $1 \times 10^{-8}\text{M}$ solution	4.5

All of these were stored in foil-wrapped bougies at 3°C.

DMSO and medium control was prepared to a 1:2000 dilution by adding 1µl DMSO to 1999µl medium. Neutral red stock solution was prepared at  $700\mu\text{g ml}^{-1}$  by adding 0.07g of neutral red powder to 100ml of PBS and stored in a sterile brown vial at 3°C. For the neutral red assay, a working solution was taken from this and made to  $70\mu\text{g ml}^{-1}$  by tenfold diluting in CHO medium. Acidified ethanol control was made by adding 50ml ethanol, 1ml acetic acid and 49ml distilled water in a glass bottle and stored in refrigerator at 3°C.

## 2.2 Neutral Red Assay

A sterile 96 well plate was set up in the configuration shown in figure 1. Each well was first seeded with cells following a method similar to a regular passage. However, two flasks of cells were emptied of medium, washed twice with PBS, trypsinised and resuspended in 5ml medium and one poured into the other due to greater number of cells needed for 96 well plate. A haemocytometer count was performed as before. This time, a concentration of  $2 \times 10^4$  cells /  $\text{cm}^3$  was required. As per figure 1, 44 wells were to be filled, each with 200 $\mu\text{l}$  of cell suspension at  $2 \times 10^4$  cells /  $\text{cm}^3$ . This required a minimum of (0.2ml x 44 wells) = 8.8ml of cell suspension. 10ml of suspension was made at a concentration of  $2 \times 10^4$  cells /  $\text{cm}^3$  to make way for error.

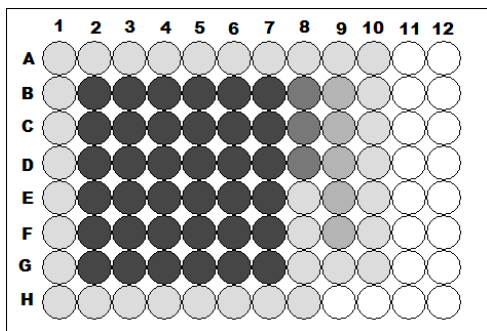


Figure 1: Configuration of 96 well microplate for Neutral Red Uptake Assay

● = BPA                      ● = Acidified ethanol  
 ● = DMSO+MEDIUM      ● = Medium

After the wells were each seeded with 200 $\mu\text{l}$  of this suspension, blank CHO medium was added to all surrounding wells to act as a heat sink. The plate was then incubated for 24 hours at 37°C.

Immediately after this, medium was removed by pipette and replaced with test substances (BPA in DMSO in medium, DMSO in medium and acidified ethanol solution for their respective wells as per figure 1). This was incubated for 48 hours at 37°C.

Following this exposure, all supernatants (except heat sink wells) were removed by pipette. 200 $\mu\text{l}$  of the 70 $\mu\text{g}/\text{ml}$  neutral red in medium solution was then added to all test wells and the plate again incubated at 37°C for three hours to allow uptake of the stain. After this, all non-heat-sink wells were drained of NR-containing medium by pipette and then washed thrice with 200 $\mu\text{l}$  PBS each. After the addition of PBS to the wells for each wash, a gentle spin of the plate for 1min was performed to assist in washing the cells. Finally, after the removal of the last washing PBS, to release the NR taken up by the cells, each well had 200 $\mu\text{l}$  acidified ethanol added to it to lyse the cells and the plate spun again for 1min to mix well. The plate was then read for absorbance at 540nm using an OPTImax tunable microplate reader (Molecular

Devices) and the results recorded using SOFTMAX software (Molecular Devices). It was decided to use one-way ANOVAs to test for significance between treatments of BPA because only one variable was in question here – the agent to which the cells were exposed. This was then used to find a point at which cytotoxicity showed a marked increase. Analyses such as this were also carried out to find differences between treatments and controls.

## 3. Results

Results of the neutral red assay and associated controls are shown in figure 2. Figure 3 shows stacked data of concentrations  $1 \times 10^{-4}\text{M}$  to  $1 \times 10^{-6}\text{M}$  BPA on the left and  $1 \times 10^{-7}\text{M}$  to  $1 \times 10^{-9}\text{M}$  BPA on the right.

Mean absorbance for  $1 \times 10^{-4}\text{M}$  BPA was 1.924 (SD 0.253, SEM 0.103). Mean absorbance for  $1 \times 10^{-5}\text{M}$  BPA was less, at 1.905 (SD 0.177, SEM 0.072).

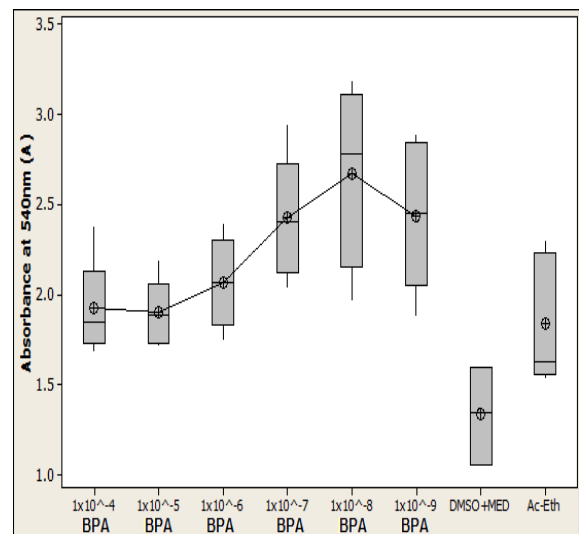


Figure 2: Boxplots of absorbance data from Neutral Red Uptake Assay. DMSO+MED = DMSO and medium control, Ac-Eth = acidified ethanol control.

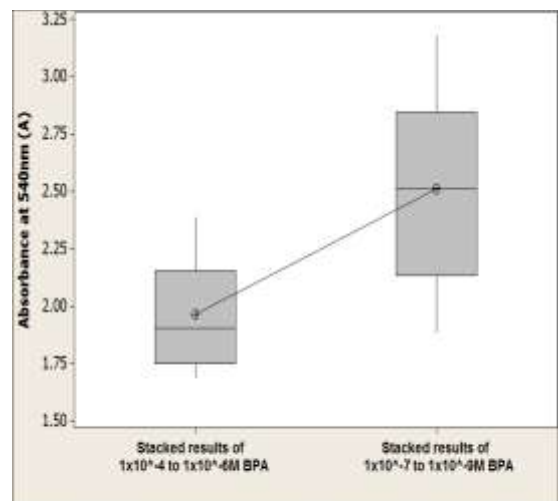


Figure 3: Boxplot of stacked results of  $1 \times 10^{-4}\text{M}$  to  $1 \times 10^{-6}\text{M}$  BPA versus stacked results of  $1 \times 10^{-7}\text{M}$  to  $1 \times 10^{-9}\text{M}$  BPA. One-way ANOVA shows clearly significant difference ( $p=0.000$ ). However, there was no statistically significant difference by one-way ANOVA within the groups ( $p=0.421$  and  $p=0.526$  for higher 3 concentrations (left boxplot) and lower 3 concentrations (right boxplot) groups respectively.)

Mean absorbance of  $1 \times 10^{-6}$  M BPA was 2.068 (SD 0.246, SEM 0.100), considerably less than that for  $1 \times 10^{-7}$  M BPA, which was 2.430 (SD 0.333, SEM 0.136). This area is where most of the interest lies. There was then only a small increase in mean absorbance of 0.239A to 2.669 for  $1 \times 10^{-8}$  M BPA (SD 0.481, SEM 0.196). The lowest concentration of BPA tested,  $1 \times 10^{-9}$  M BPA, had a mean absorbance of 2.435 (SD 0.396, SEM 0.162), which is actually less than the previous mean absorbance by 0.234A. There is, however, no statistically significant difference here between  $1 \times 10^{-8}$  M BPA treatment and  $1 \times 10^{-9}$  M BPA treatment ( $p=0.381$ ).

The DMSO + medium control had a mean absorbance of 1.333 (SD 0.272, SEM 0.157), which is statistically significantly lower than all the means for the BPA treatments ( $p=0.001$  for a one-way ANOVA between stacked results of all BPA treatments versus the DMSO + medium control.) The acidified ethanol control had a mean absorbance considerably higher than that of the DMSO + medium control by a margin of 0.509A, coming out at 1.842 (SD 0.360, SEM 0.161).

There was no statistically significant difference between the concentrations of  $1 \times 10^{-4}$  M to  $1 \times 10^{-6}$  M BPA ( $p=0.421$ ). Neither was there a significant difference between  $1 \times 10^{-7}$  M to  $1 \times 10^{-9}$  M BPA ( $p=0.526$ ).

There was a highly statistically significant difference between the two measured extreme concentrations of  $1 \times 10^{-4}$  and  $1 \times 10^{-9}$  M BPA ( $p=0.024$ ), satisfying the 95% confidence interval. When results are taken as groups, there was an extremely statistically significant difference between  $1 \times 10^{-4}$  to  $1 \times 10^{-6}$  M BPA and  $1 \times 10^{-7}$  to  $1 \times 10^{-9}$  M BPA ( $p=0.000$ ), satisfying the 99.9% confidence interval (stacked one-way ANOVA of  $1 \times 10^{-4}$  to  $1 \times 10^{-6}$  M BPA versus  $1 \times 10^{-7}$  to  $1 \times 10^{-9}$  M BPA shown in figure 3.)

A significant difference exists also between  $1 \times 10^{-5}$  M and  $1 \times 10^{-8}$  M BPA ( $p=0.004$ ). Significance is not found, however, between  $1 \times 10^{-6}$  M and  $1 \times 10^{-7}$  M BPA ( $p=0.058$ ) as this is just short of the 95% confidence interval.

It is worth noting that there is a noticeable difference in the boxplot between the DMSO + medium control and the acidified ethanol control. These are not statistically significant ( $p=0.081$ ), falling short of the 95% confidence interval (figure 2.) Also it is worth noting that the DMSO + medium control shows greatly decreased absorbance compared to all BPA treatments and even acidified ethanol. Possible reasons for this, both experimentally and toxicologically, will be elaborated upon in the discussion.

#### 4. Discussion

There is a clear progression from lower absorbances (lower cellular viability) to higher absorbances (higher cellular viability) as the concentration of BPA decreases from left to right in figure 2. The first main point of interest however, lies in discerning at what level there is greatest change in cellular viability and therefore at what level cells begin to experience the largest drop in viability for the smallest increase in concentration. This could be seen as

the point of tolerance or toxicity "threshold". The second main point of interest is in discerning to what level even the lower concentrations of BPA affect cellular viability compared to control.

In order to find out at what point there is greatest increase in cytotoxic effect of BPA for the smallest increase in BPA concentration, the lowest and highest concentrations used were compared for statistically significant difference and were found to be statistically different ( $p=0.024$ ). Then the next highest and next lowest would be compared until there was no statistically significant difference between the cytotoxic effect of those concentrations, at which point it could be said that around or between those two concentrations lay the point of greatest cytotoxic effect for smallest change in concentration. Comparison of  $1 \times 10^{-5}$  M and  $1 \times 10^{-8}$  M BPA revealed highly significant difference also ( $p=0.004$ ). It was only when our middle two concentrations of  $1 \times 10^{-6}$  and  $1 \times 10^{-7}$  were compared that we were unable to satisfy a 95% confidence interval ( $p=0.058$ ).

The significance level drops below the 95% confidence interval only when making comparison between the  $1 \times 10^{-6}$  M and  $1 \times 10^{-7}$  M BPA measurements; it is therefore in that range where differences become more difficult to discern. It is also visible from the boxplots in figure 2 that the greatest change in toxicity exists between the  $1 \times 10^{-6}$  M and  $1 \times 10^{-7}$  M BPA tests as visible by the large gradient of the line between the two. It is therefore reasonable to suggest that this is indeed the point at which the smallest increase in concentration produced the biggest increase in cytotoxicity. Further experiments with smaller differences in concentration, but all within the  $1 \times 10^{-6}$  M and  $1 \times 10^{-7}$  M BPA range could continue to pinpoint the concentration at which these mammalian cells start to exhibit a pronounced reduction in cell viability. It is clear to see, however, that, in conclusion, BPA does indeed have cytotoxic effect, observable in vitro, and that even low concentrations of around  $1 \times 10^{-6}$  M to  $1 \times 10^{-7}$  M BPA have a pronounced effect on cell viability.

It is unfortunate, however, that the absorbances for the control DMSO + medium were so unexpectedly low (for reasons hypothesised below) as this would have allowed for all results to be compared to a "healthy" control. Whilst it was known that DMSO has a weakly cytotoxic effect, it was completely unanticipated that the control would give such an outlying result.

Regarding such apparent high cytotoxicity of DMSO + medium controls, the exact cause of this unexpected reading is unknown. It was expected that DMSO + medium would produce higher absorbances (better cell viability) than all BPA exposures and acidified ethanol 100% lysis control.

Whilst the experiment's meaning remains intact regarding the cytotoxic effects of relative concentrations of BPA, it is suggested that the experiment be repeated with many more repeats of the DMSO + medium control. This would give a much more definite answer than just the three that were available here. There was also some concern over

the expansion of DMSO when exposed to the incubator's temperature. It is particularly strange that the acidified ethanol control seemed to produce far less cellular lysis than expected. Whilst the acidified ethanol stock solution was left in the refrigerator for some time, it was not considered to be one of the agents that would deteriorate.

Therefore it would be worth repeating the DMSO + medium controls with a far greater number of repeats and away from all other wells in case of expansion overflow and also to perform fresh acidified ethanol lysis of the same concentration of seeded CHO-K1 cells in wells to ascertain whether or not this reading is an anomaly, due to settling or deterioration of stock solution or some other mechanism.

Finally, one last area of improvement for studies such as this would be to use non polycarbonate wares for all of the experiment to eliminate BPA leaching. As a great deal of equipment here (most notably, the vials and microplates) were made of polycarbonate plastic, there will most likely have been some leaching of BPA from the equipment. Whilst this can be ignored to some extent due to the fact that the same equipment was used under the same conditions for all of the work, there may well have been some effect as this work's very nature elucidates that BPA has cytotoxic effects in-vitro at even very low concentrations. The presence of a solvating agent such as DMSO may well have added to the leaching as solvents are known to increase the rate of BPA leaching (Krishnan et al., 1993.)

In conclusion, there are far wider implications of this study than simply that BPA is cytotoxic at low concentrations. A re-evaluation of the safety of polycarbonate plastic is in order. At such low concentrations of BPA as  $1 \times 10^{-6}$ M (micromolar), it is clear to see, coupled with the fact that polycarbonate plastics and BPA leachates are all around us, that this can have a definite and direct cytotoxic effect. It is worth noting, however, that published literature cited here has not found amounts of BPA present in human fluids that cross the putative "threshold of toxicity" found here. Further studies, particularly with the addition of more controls, are in order to more accurately elucidate the exact effects at different concentrations.

What most definitely is cause for concern, however, is the amount of BPA leached from aging polycarbonate plastic. The study by Howdeshell et al (2003) clearly shows a value of  $310 \mu\text{g/L}$  leaching from aged polycarbonate animal cages into distilled water. This concentration is above the "threshold of toxicity" as it corresponds to  $1.36 \times 10^{-6}$ M BPA. Therefore, rather than concentrating on reassuring statistics that show low physiological levels of BPA, studies must begin into which polycarbonate plastic products (including dental resins as BPA leaches from these also (Takahashi & Oishi, 2000)) and their BPA leaching curves. For example, there may well become a point in the lifetime of certain products at which the levels of BPA leaching cross this threshold of toxicity.

In addition to the known oestrogenicity of BPA, the cytotoxic effects of directly ingesting such levels of BPA

are as yet unknown. More studies need conducting into how BPA is absorbed as, while serum levels and urine levels may seem low at the moment, there may be much higher levels directly acting on the gastrointestinal tract as this is what is in contact with ingested fluids, for example. This could, in turn lead to increased risk of GIT pathology. This may also be true of the liver which is responsible for the conjugation of BPA and may well accumulate there at higher levels than found in serum.

This becomes even more concerning when bearing in mind that levels of BPA shown to be cytotoxic will be released in relatively large amounts from polycarbonate baby bottles and these are warmed for the baby, further increasing BPA leaching. Due to the reduced capacity of babies (by pure lack of body mass) to eliminate BPA, the cytotoxic effects of this compound may well be aggravated due to the well-appreciated dose/body mass relationship. These thoughts make the cytotoxicity of these very low concentrations of BPA all the more worrying and strengthen existing calls for a thorough investigation into the distribution of BPA presence throughout the human body. This must be joined by studies into the leaching of BPA from products that will cause ingestion/exposure to BPA and the product age/BPA-leaching relationship and a more extensive investigation into the occurrence of BPA in our environment and therefore its effects on the wider ecosystem.

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