Xenoestrogen Bisphenol-A's Neurotoxicity via Estrogenic Activity and Resulting Alzheimer's Disease Pathogenesis

(Research Paper)

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I. INTRODUCTION

1.1 Alzheimer's Disease: Background

The most common form of dementia, Alzheimer's Disease (AD) is a debilitating neurodegenerative disease and known for its gradual memory loss. The disease begins as early stage-forgetfulness, followed by worsening of language and memory, and eventually severe loss of bodily function, resulting in death (Wang & Reddy, 2017). In fact, (Bali et al., 2012) predicts that AD incidence will rise to 1 in 85 globally by 2050. It is imperative to learn more about preventative mechanisms

1.2 Two Origins of AD

Familial AD (FAD) arises due to the genetic mutations of presenilin-1, presenilin-2 d, and amyloid precursor protein (APP) genes, which are transmissible from parent to child. Individuals who possess these mutations are more susceptible to developing FAD (Wainaina, Chen, and Zhong, 2014).

The other 95% of AD cases is **sporadic AD (SAD), or Late-Onset Alzheimer's Disease (LOAD)**. Manifestation of Late-onset AD involves a combination of genetic mutations, environmental factors, and lifestyle choices (Wainaina, Chen, and Zhong, 2014). However, what is more puzzling is that the specific molecular mechanisms of sporadic AD, Late-Onset Alzheimer's Disease (LOAD), have not yet been agreed on; however, many conjectures regarding the mechanism have been made. Numerous genetic families have been suggested as potential causes for late-onset AD (Blennow & Zetterberg, 2018; Wainaina, Chen, and Zhong, 2014). However, genetic expression can only partially explain late-onset. Environmental factors still play an important part in the expression of these genes . For example, it has been postulated that the dysfunction of the Hypothalamic-pituitary-adrenal axis (HPA axis) is the main system in which of the symptoms that arise during AD development. Regulation of intracellular events, such as inflammation, cytotoxicity of extracellular Aβ peptides, and immune cell activation, may be the more specific mechanisms or pathways in which AD can be induced (Blennow & Zetterberg, 2018; Lawrence, 2009; Wainaina, Chen, and Zhong, 2014).

This study investigated the influence of bisphenol-A, an environmental pollutant, on the pathophysiological formation of AD at cellular and molecular level.

1.3 Amyloid-Tau Neuropathy

Amyloid-beta plaque deposits (extracellular)

Although FAD and SAD originate from two different causes—FAD primarily relies on PS1, PS2, and APP gene families (Blennow & Zetterberg, 2018), while SAD is a result of genetic-environment factor interactions—, cases of Alzheimer's exhibits the classic extracellular Aβ deposits and intracellular tau-hyperphosphorylated neurofibrillary tangles (Bali et al., 2012). The APP protein is cleaved by the β-secretase and γ-secretase enzyme. What remains is usually a 42 amino acid peptide called Aβ monomer (Wang & Reddy, 2017). When these monomers accumulate, they oligomerize into extracellular Aβ insoluble plaques, which cause loss of long term potentiation, synapse damage (which interferes with neuronal communication), and neuronal cell death. In other words, Aβ plaques are toxic to cells and results in neuronal dysfunction.

In FAD, the APP cleavage results from the genetic mutations. In SAD, the initial trigger for the cleavage is unknown. However, as (Wainaina, Chen, and Zhong, 2014) have suggested, these plaques are of extracellular origin and therefore may be influenced by environmental factors.

1.4 Bisphenol-A: Brief overview

Bisphenol-A, or BPA, is a chemical additive used by manufacturers in the production of polycarbonate plastics and epoxy resins (Inadera, 2015). It is often found in food containers, plastic water bottles, canned goods, toys, and even medical dental products (Wang et al., 2019).

Because of its ability to maintain structural integrity and flexibility at the same time, its demand by manufacturing industries has grown exponentially for the past 20 years (Lee et al., 2016). Consequently, the exposure of BPA ubiquitous environmental pollutant that permeates through

every aspect of an industrialized society in terms of manufactured products, has the potential to disrupt the homeostasis of systems relevant to the pathogenesis of AD.

1.5 Bisphenol-A: Toxic Effects

BPA can cross the blood-brain barrier. In growing fetuses, the blood-brain barrier is especially susceptible ("leaky") to outside contaminant exposure via maternal circulation. The damage done by BPA on developing children are almost irreversible (Birla et al., 2019). Developmental abnormalities and behavioral changes are evident in exposed infants and children, respectively (Birla et al., 2019).

Nevertheless, BPA, an endocrine disrupting compound (EDC), is known for its estrogenic activity. Depending on cell type, concentration, and 17B-Estradiol, BPA can exhibit different degrees of antagonism or agonism on the estrogen receptor (Rogers, Metz & Yong, 2013). Thus, it is necessary to first study its effects on the cells used for the experiment. BPA's interference with the estrogen receptor signaling due to its interaction with the estrogen receptor can interfere with Ca2+ fluxes used in signaling and maintaining neuronal health (Lee et al., 2008). Since Aβ oligomer accumulation interacts with neuronal membranes and forms pores with Ca2+ selectivity, BPA may aggravate AD progression and maybe even increase the risk for AD inducement (Wainaina, Chen, and Zhong, 2014) (Wang et al., 2016).

Moreover, studies have shown that women over 60 have greater risk than men to develop Alzheimer's (Imtiaz et al., 2017; Janicki & Schupf, 2010), indicating estrogen signaling mechanism may be related to AD and that BPA's estrogenic activity possibly plays a role in AD onset and pathogenesis.

For these reasons, BPA is a viable candidate as an environmental factor that aggravate AD or induce neurodegeneration, which increases the chances of AD pathogenesis. It is ubiquitous in the environment, can cross the blood-brain barrier, and interacts with estrogen receptors, which is why BPA must be investigated for specific neurological impact like AD.

II. MATERIALS & METHODS

2.1 Cell Culture

The HTB-11 human neuroblastoma cell line (ATCC, New York, NY) was grown *in vitro* at 37 degrees Celsius in Eagle's Minimum Essential Medium (MEM) and 10% fetal bovine serum (Invitrogen, USA). HTB-11 cells serve as a neuronal cell model to analyze the impact of BPA on neuronal cell health and interaction with amyloid beta neurotoxicity.

The RAW 264.7 Murine Macrophage cell line (ATCC, New York, NY) was cultured *in vitro* at 37 degrees in Dulbecco's Modified Eagle's Medium with 10% fetal bovine serum (Invitrogen, USA). RAW macrophages were used to study BPA's potential to induce neuroinflammation.

2.2 Preparing Cell Plates

HTB-11 cells were cultured in a flask at 37 degrees. Excess media was decanted and the adherent cells were trypsinized to initiate detachment from the flask in order to extract cells. After 4 minutes of incubation, 5 mL of media was added and thoroughly mixed with the cells. The solution was subsequently transferred to a 15mL tube and centrifuged for 4 minutes at 2000 rpm to aggregate a concentrated cell pellet. The waste media was discarded and replaced, and the cells were homogenized into new media with a vortexer. 100µL was added to each well in a 96 well plate and then incubated at 37 degrees Celsius for 24 hours, allowing for cellular adhesion.

2.3 Creating Dilutions

A stock solution of BPA was created with a 50% water and 50% dimethyl sulfoxide (DMSO) solvent. DMSO was necessary to help BPA dissolve in solution. Further dilutions were made in cell culture media to achieve concentrations of 2µM, 20µM, and 200µM. DMSO concentration was calculated and used in the control wells to eliminate confounding variable of DMSO's toxicity.

2.4 MTT Assay

MTT assay is a colorimetric assay that can assess the cell metabolic activity, which is an indicator of cell viability. Cells are incubated first with BPA treatment for 24 hours and then

incubated for 2 hours with yellow MTT tetrazolium dye. The NAD(P)H-dependent cellular oxidoreductase enzymes in a living cell will reduce a yellow tetrazolium dye into purple, insoluble formazan. Dimethyl Sulfoxide (DMSO) was subsequently added to break apart (and thus killing) all of the cells to release the reduced MTT. The 96-well plate was read by a microplate reader at a wavelength of 550 nm, because the absorbance of the plate reflects the cell viability. The absorbance values are compared to control values to generate the survival rate for each treatment. All assays were performed in triplicate. The MTT assays were used to assess viability in both HTB-11 cells and RAW 264.7 cells.

2.5 Enzyme-Linked Immunosorbent Assay (ELISA)

To study the protein expression of cells, this project used an ELISA to determine protein levels. The first set of steps is diluting the stock solution from the ELISA kit (Boster Biological Technology, CA) and setting up standards in eight tubes. Standards are known protein quantities that will be used as a reference value; results obtained from treatment will be compared to the standards. In preparing the samples, the cell culture must be cleared of media and then stored at -20 degrees Celsius. Before using, bring all the reagents to 37 degrees. The wash powder was then dissolved in 1000mL of water to create the 1X PBS wash buffer.

For Biotinylated Anti-Human IL1B antibody reagent, prepare this reagent immediately prior to use by diluting the Human IL1B Biotinylated antibody (100x) 1:100 with Antibody Diluent. Prepare 100 microliters by adding 1 microliter of Biotinylated antibody (100x) to 99 microliters of Antibody Diluent for each well. Mix gently and thoroughly and use within 2 hours of generation. For Avidin-Biotin-Peroxidase Complex reagent, prepare this reagent immediately prior to use by diluting the Avidin-Biotin-Peroxidase Complex (100x) 1:100 with Avidin-Biotin-Peroxidase Diluent. Prepare 100 microliters by adding 1 microliter of Avidin-Biotin-Peroxidase Complex (100x) to 99 microliters of Avidin-Biotin-Peroxidase Diluent for each well. Mix gently and thoroughly and use within 2 hours of generation. For Human IL1B reagent, the standards are to be prepared no more than 2 hours prior to performing the experiment. Use one 1 ng of lyophilized Human IL1B standard for each experiment. Gently spin the vial prior to use. Reconstitute the standard to a stock concentration of 1 ng/mL using 1 mL of sample diluent.

Assay Protocol From (Boster Biological Technology, CA):

1. Prepare all reagents and working standards as directed previously.
2. Remove excess microplate strips from the plate frame and seal and store them in the original packaging.
3. Add 100 microliters of the standard, samples, or control per well. Add 100 microliters of the sample diluent buffer into the control well (zero well). At least two replicates of each standard, sample, or control is recommended.
4. Cover with the plate sealer provided and incubate for 120 minutes at RT (or 90 min. At 37 degrees C).
5. Remove the cover and discard the liquid in the wells into an appropriate waste receptacle. Invert the plate on the benchtop onto a paper towel and tap the plate to gently blot any remaining liquid. It is recommended that the wells are not allowed to completely dry at any time.
6. Add 100 microliters of the prepared 1x Biotinylated Anti-Human IL1B antibody to each well.
7. Cover with plate sealer and incubate for 90 minutes at RT (or 60 minutes at 37 degrees C).
8. Wash the plate 3 times with the 1x wash buffer.
9. Add 100 microliters of the prepared 1x Avidin-Biotin-Peroxidase Complex into each well. Cover with the plate sealer provided and incubate for 40 minutes at RT (or 30 minutes at 37 degrees C)
10. Wash the plate 5 times with the 1x wash buffer.
    a. Discard the liquid in the wells into an appropriate waste receptacle. THen, invert the plate on the benchtop onto a paper towel and tap the plate to gently blot any remaining liquid. It is recommended that the wells are not allowed to completely dry at any time
    b. Add 300 microliters of the 1x wash buffer to each assay well. (For cleaner background incubate for 60 seconds between each wash)
    c. Repeat steps a-b 4 additional times.

11. Add 90 microliters of Color Developing Reagent to each well. Cover with the plate sealer provided and incubate in the dark for 30 minutes at RT (or 15-25 minutes at 37 degrees C). (The optimal incubation time must be empirically determined. A guideline to look for is blue shading the top four standard wells, while the remaining standards remain clear.)
12. Add 100 microliters of Stop Solution to each well. The color should immediately change to yellow.
13. Within 30 minutes of stopping the reaction, the O.D. absorbance should be read with a microplate reader at 450 nm.

3. RESULTS

To elucidate a possible mechanism (Estrogen receptor signaling) by which BPA interferes with, BPA was co-incubated with different substances; cell viability rates were determined from the data.

3.1 BPA's Effect on neuronal cells

3.1.1 BPA's Effect on Human Neuronal Cell Proliferation

HTB-11 neuronal cell survival rates measured with the MTT cell viability assay. All concentrations of BPA significantly decreased the survival rates. Higher concentrations displayed significantly lower survival rates than lower concentrations of BPA, indicating that higher concentrations are more cytotoxic in dose dependent manner (fig 1).

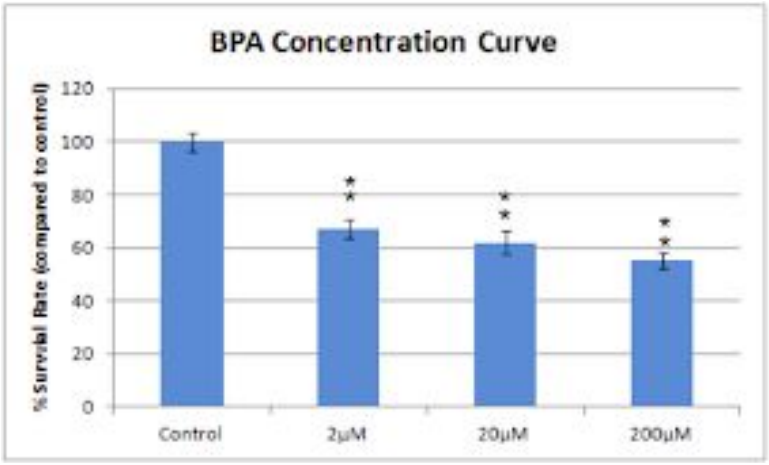

Fig 1

Cell survival rate of HTB-11 cells after 24 hour treatment of BPA at 2μM, 20μM, or 200μM was measured via absorbance value readings. Readings from identical treatments in two different cell plates

were averaged. Averages were all compared to 2μM killed 32.9% of cells, 20μM killed 38.11% of the cells, and 200μM killed 56.2% of the cells. Stars represent significance to control ($p<0.05$*,$p<0.01$**, $p<0.001$***)

3.1.2 BPA Enhances Amyloid-beta Cytotoxicity

Amyloid beta peptide treatment yielded 62.28% survival rate) and is toxic alone, and BPA at all concentrations are cytotoxic: The 2μM BPA treatment killed 32.9% of cells (survival rate of 67.1%, $p<0.01$) and 20μM BPA killed 38.11% of cells (61.89% survival rate, ($p<0.01$, compared to control). Co-incubation of Aβ42 and BPA significantly kills more cells and therefore decreases neuronal cell survival rate (fig 4), demonstrating that BPA exacerbates amyloid-beta neurotoxicity. Similar to BPA alone, the co-incubation treatments also exhibited a dose-response effect: 2μM + Aβ42 killed 65.72%, whereas 20μM + Aβ42 killed 84.46% of the cells. Indeed, 20μM + Aβ42 had a significantly lower survival rate. There is also a greater significant difference between the 2μM+Aβ and 20μM +Aβ ($p<0.001$) than there was between 2μM and 20μM ($p<0.05$).

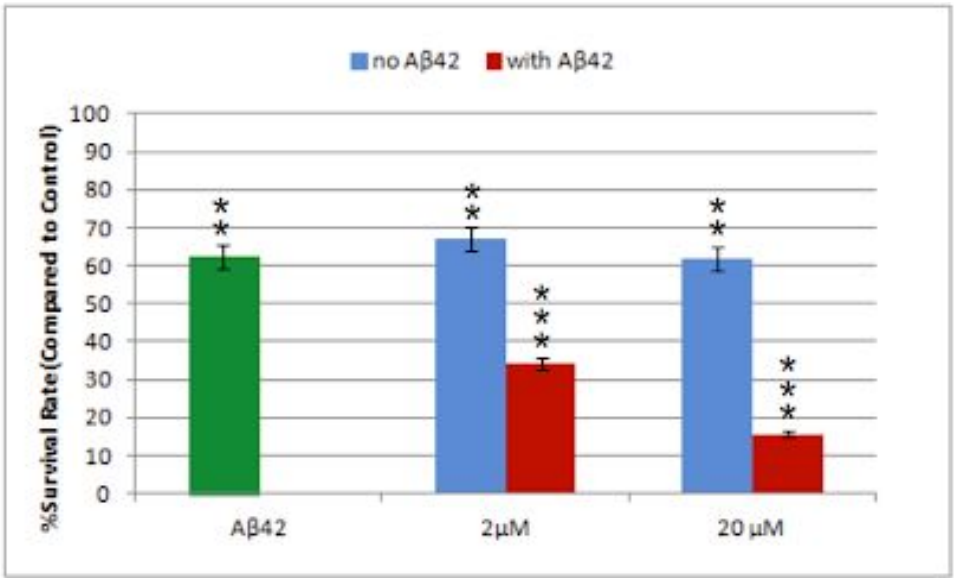

Fig. 2

A cell viability assay was run to give absorbance readings for each cell well. All well readings in a treatment (regardless of plate) were averaged; the average value was then compared to control, generating the survival rate. Amyloid beta peptides and BPA individually both induce neurotoxicity and exhibit a synergistic effect when combined (green bar=Amyloid beta treatment only, blue bars=BPA only, red

bars=contains both BPA and amyloid beta) Stars represent significance to control ($p<0.05$*,$p<0.01$**, $p<0.001$***)

3.1.3 BPA's Effect on Amyloid Precursor Protein Expression in Neuronal Cells

BPA-induced APP expression levels were compared to the control group to determine percent increase. BPA treatments exhibit significant increase in APP expression. Furthermore, the 20µM BPA exhibited 25.84% higher APP expression, while 2µM BPA increased APP expression by 8.10% (fig 5), demonstrating that higher concentrations of BPA have greater APP expression and thus a dose-response effect of BPA on APP expression exists.

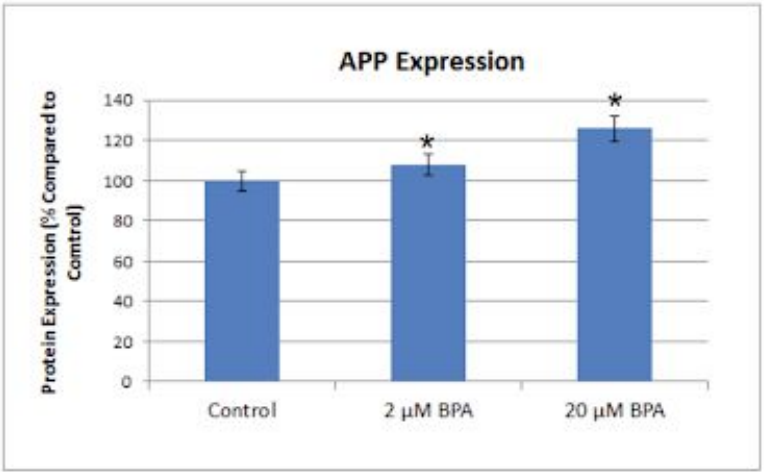

Fig 3

There was a significant percent increase in Amyloid Precursor Protein (APP) expression induced by BPA treatments after 24 hour incubation. APP was measured because cleavage of this protein leads to amyloid beta plaque formation. An ELISA was performed, and absorbance readings were taken from the wells. A regression line was created using absorbance readings to predict APP levels in the wells. Every well had absorbance readings and therefore protein levels; the control group was used as a baseline number to compare the BPA treatments. Stars represent significance to control ($p<0.05$*,$p<0.01$**, $p<0.001$***)

3.2 BPA's Effect on Immune Cells

3.2.1 BPA's Effects on Immune Cell Proliferation

Both BPA concentrations significantly decrease the survival rate. According to figure 6, 20µM BPA killed more cells (55.13% survival rate) than 2µM did (85.86% survival rate),

demonstrating that BPA concentrations have dose-dependent cytotoxicity on macrophages tested.

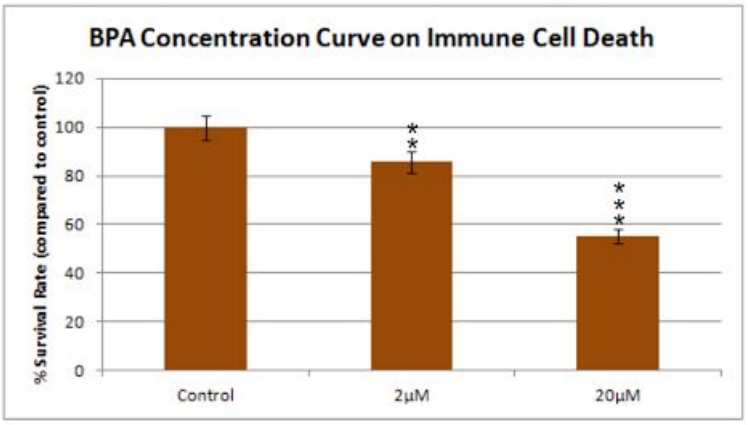

Fig 4

The MTT cell viability assay was used to obtain absorbance values. The values of one treatment in all wells were averaged and then compared to control, generating the cell survival rate. The graph above displays the concentration curve for RAW 264.7 (macrophage) survival rate after incubation for 24 hours. The higher concentration of BPA killed more cells (55.13% survival rate) than the lower concentration did (85.87% survival rate). Stars represent significance to control ($p<0.05$*,$p<0.01$**, $p<0.001$***)

3.2.2 BPA's Effect on Immune Cell Expression of Pro-Inflammatory Cytokines

3.2.2.a IL-1β Cytokine Expression

BPA significantly increases interleukin-1beta (IL-1beta) expression, which appears to be dose-responsive, as the 20μM BPA has significantly greater or more abnormal IL-1beta expression than that of 2μM BPA: the lower concentration (2μM BPA) increased protein expression by 36.62%, while the higher concentration (20μM BPA) increased it by 53.80%.

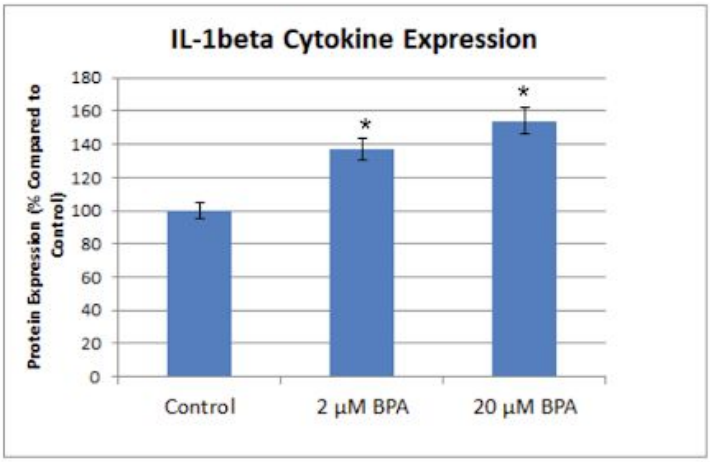

Fig 5

An ELISA was performed to obtain absorbance values of the wells. The values for one treatment (regardless of plate) were averaged. A regression was created using all the values to predict IL-1beta expression levels. Expression of this cytokine was measured to give insight into the inflammatory process. Incubation with BPA concentrations for 24 hours induced significantly higher IL-1beta expression. Stars represent significance to control ($p<0.05$*,$p<0.01$**, $p<0.001$***)

3.2.2.b TNF-alpha Cytokine Expression

All BPA concentrations displayed cytokine expression levels significantly greater than that of control. The expression of TNF-alpha cytokines exhibited a dose-response effect, where a higher concentration (20µM BPA) induced higher levels of TNF-alpha than 2µM did.

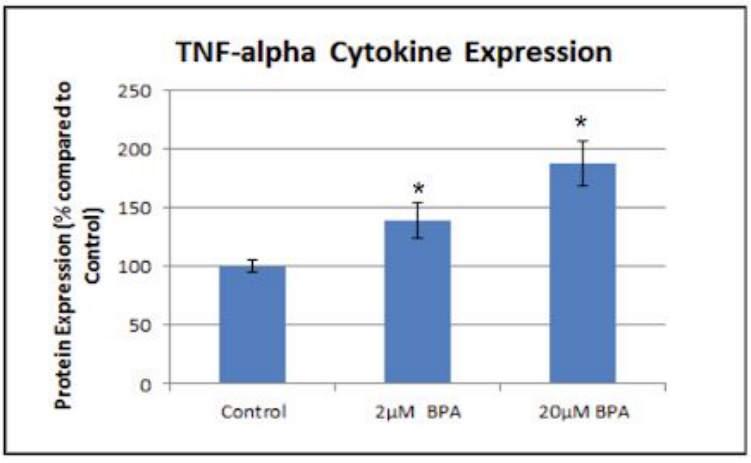

Fig 6

An ELISA was performed to obtain absorbance values of the wells. The values for one treatment (regardless of plate) were averaged. A regression was created using all the values to predict

TNF-alpha expression levels. Expression of this cytokine was measured to give insight into the inflammatory process. Incubation with BPA concentrations for 24 hours induced significantly higher TNF-alpha expression. Stars represent significance to control ($p<0.05$*,$p<0.01$**, $p<0.001$***)

3.3 BPA's Estrogenic Activity via Modulation with the Estrogen Receptor

3.3.1 Tamoxifen enhanced BPA Cytotoxicity

Tamoxifen (TAX), an estrogen receptor antagonist, alone had a survival rate of 80.5% and thus does not demonstrate severe cytotoxicity compared to BPA. However, when TAX was co-incubated with BPA for 24 hours, it decreased survival rate even further for 2µM BPA (from 67.1% alone to 48.32% with combination) and 20µM BPA (from 61.89% alone to 40.30% with combination), but it did not significantly alter survival rate of 200µM BPA (52.89% survival rate) ($p>0.05$, compared to 200µM). Results suggest an additive effect between TAX and BPA for 2µM and 20µM BPA, but not 200µM BPA.

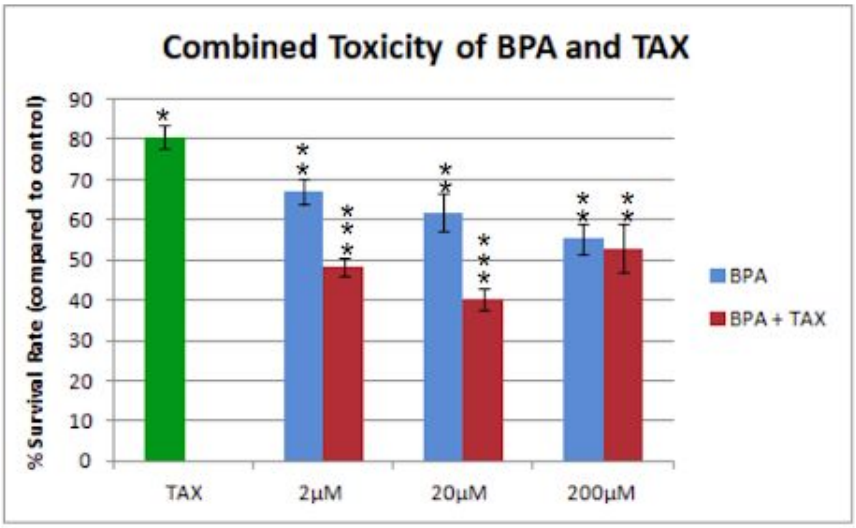

Fig 7

A cell viability assay was used to obtain absorbance readings, which were then compared to control to generate a cell survival rate. Tamoxifen, an estrogen receptor antagonist, was added to determine if BPA interacts with the estrogen receptor. Individual BPA treatments displayed a dose-response effect, while the BPA+TAX exhibited a non-monotonic dose response curve. Stars represent significance to control ($p<0.05$*,$p<0.01$**, $p<0.001$***)

3.3.2 BPA's Modulation with the Estrogen Receptor

Co-incubation of 17beta-estradiol (E2) with 2µM BPA did not significantly change the survival rate of 2µM-alone group. However, co-incubation of E2 with 20µM BPA yielded a 79.38% survival rate, which is significantly higher than that of 20µM BPA alone (55.14% survival rate). Moreover, 2µM + E2 and 20µM + E2 killed similar percentages of cells; there were no significant differences between their respective survival rates. A dose-response effect was not observed for BPA+E2.

When 2µM, E2, and TAX were all incubated together, the treatment killed 79.5% of the cells (survival rate is 20.5%). When 20µM, E2, and TAX were all incubated together, the treatment killed 82.00% of the cells (survival rate is 18.00%). There is no significant difference between the two combinations. Similarly, there is no significant difference between 2µM + E2 (81.68% survival rate) and 20µM +E2 (79.38% survival rate). A dose-response effect was not observed for BPA + E2 + TAX.

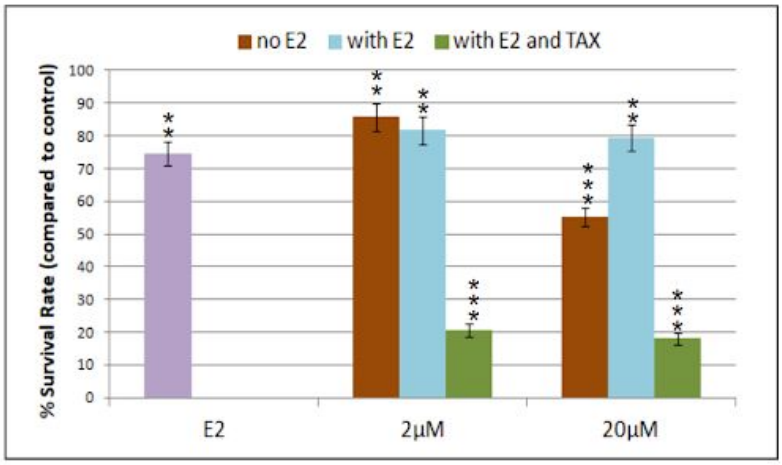

Fig 8

A cell viability assay was performed to assess survival rates. E2, 17beta-estradiol or estrogen, was used because it binds to the estrogen receptors. TAX, tamoxifen, was tested because it is an estrogen receptor antagonist. Survival rates of combinations were significantly less than control, but difference in concentrations did not induce significant changes. Stars represent significance to control ($p<0.05$*, $p<0.01$**, $p<0.001$***)

3.3.3 BPA enhances Amyloid-beta Toxicity on Immune Cells

According to figure 8, the amyloid beta peptide and BPA concentrations combined kill more cells than the two chemicals alone. In terms of individual treatments, AB42 killed off the most cells and produced a 64.67% survival rate ($p<0.01$, compared to control) and was significantly different from individual treatments of 2µM BPA ($p<0.05$) and 20µM BPA ($p<0.05$). The combinations kill more cells than just the chemicals alone. The combination of 2µM BPA + AB42 had a survival rate of 56.23%, while the 20µM BPA+AB42 had a lower survival rate of 50.12%. Both individual treatments and combinations follow a dose-response curve, where the higher the BPA concentration, the greater number of cells the combination killed, and thus, the lower the survival rate. This indicates that there is a possible additive effect between amyloid beta peptides and BPA in the macrophages.

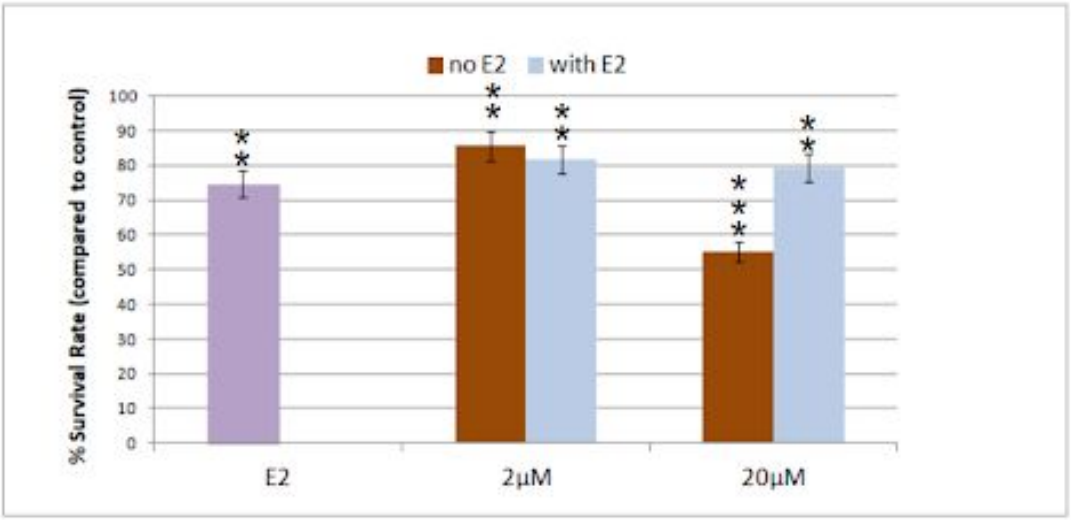

Fig 9

The graph above displays the cell survival rates after cells are incubated with AB42, BPA, or both. A cell viability assay was used to determine absorbance values and when compared to control, generated survival rates. Amyloid beta peptides and BPA concentrations combined appeared to have an additive or worsened effect. Stars represent significance to control ($p<0.05$*,$p<0.01$**, $p<0.001$***)

3.3.4 BPA-Amyloid-beta Toxicity in the Presence of 17 beta-Estradiol and Tamoxifen

Amyloid beta peptides are cytotoxic, as indicated by the significantly lower survival rates. Amyloid-beta 42 toxicity increases significantly (compared to amyloid beta-induced toxicity alone) when it is combined with a BPA concentration, E2, and/or TAX. All the combinations killed a similar number of cells, yielding similar survival rates. Only AB42+2µM BPA + TAX is significantly different from the other combinations. All other combinations are not significantly different from each other.

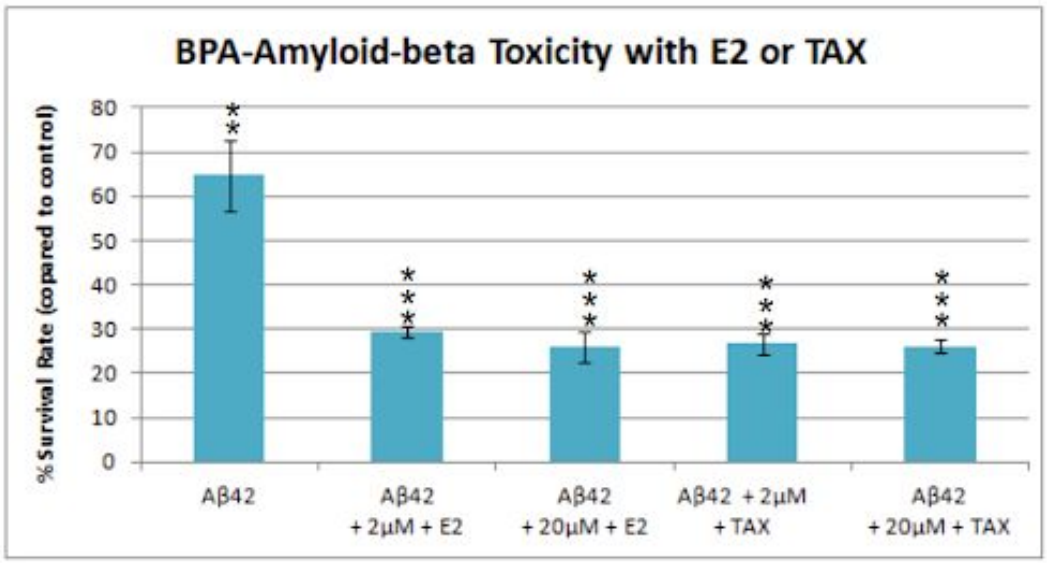

Fig 10

A cell viability assay was used to test combination effects. Absorbance readings were obtained; all readings of the same treatment were averaged and then compared to control. There appears to be no noticeable dose-response effect. All combinations have a survival rate of approximately 25% to 30%. Stars represent significance to control ($p<0.05$*,$p<0.01$**, $p<0.001$***)

4. DISCUSSION

4.1 BPA increases risk for neurodegeneration

A few studies have examined and suggested that BPA has deleterious effects on material (i.e. DNA and chromosomes) in normal body cells (Inandera, 2015). It was observed that BPA has induced loss of neuronal cells, with survival rates dependably under 70% for all concentrations. This suggests that BPA is cytotoxic to neuronal cells, and since neurodegeneration is the loss of

structure and function of neurons, BPA therefore has possible neurodegenerative potential at 2μM, with a dose-effect seen at increasing concentrations.

4.2 BPA enhances Amyloid-beta toxicity

BPA alone not only significantly decreases the survival rate of neurons, but it also exacerbates Aβ cytotoxicity in neurons. Neuronal cells treated with BPA + Aβ experienced a remarkable loss of neurons and low survival rate compared to the treatments alone, suggesting an additive effect between the two chemicals. With these levels of cell death, BPA can accelerate neurodegeneration or aggravate the pathogenesis of Alzheimer's Disease, demonstrated in the experiment.

Additionally, BPA can also promote elevation of Amyloid Precursor Protein (APP). Using various secretases, APP can be cleaved into peptides of 38, 40, or 42 amino acids (Qiu, Liu, Chen, Zhao & Li, 2015) (O'Brien & Wong, 2011). The Aβ42 peptide is most commonly implicated in the toxic oligomerization into and accumulation of plaques and later the formation of amyloid fibrils (Qiu, Liu, Chen, Zhao & Li, 2015). These will disrupt synaptic function and therefore significantly hinder the neuronal cell communication. This partially can explain the memory loss and general confusion of patients with Alzheimer's. The higher the level of APPs, the more cleaving can occur, and the higher the chance that plaques will form (O'Brien & Wong, 2011). Thus, BPA'-induced elevation of APP levels implicates BPA promoting Alzheimer's disease progression.

Furthermore, only the initial plaque formation and deposition is needed to trigger Alzheimer's onset. The extracellular amyloid beta plaques trigger internal tau hyperphosphorylation (Qiu, Liu, Chen, Zhao & Li, 2015). The hyperphosphorylation creates neurofibrillary tangles (NFTs) and disrupts internal transport of essential molecules and nutrients (Wang et al., 2016). The neurons eventually die if the NFTs are too severe (Wang et al., 2016). Thus, BPA's initial trigger or interference in body systems can aggravate AD and start a feedback loop of NFTs and Aβ plaques, perhaps even prompting AD onset.

4.3 BPA induces neuroinflammation through pro-inflammatory cytokine disruption

BPA treatment significantly decreased survival rate in macrophages, supporting that BPA is cytotoxic to immune cells. As expected, an increase in concentration of BPA led to an increase in the number of cells killed (or a decrease in cell survival rate). This suggested immune cytotoxicity of BPA has serious implications in AD. BPA-induced macrophage death leaves fewer amounts of macrophages to clear existing or future insoluble Aβ plaques formation (Li et al., 2018). The plaques are currently implicated as the biggest factor in causing AD symptoms. Lack of clearing also increases the chance for Aβ plaque accumulation.

Extracellular Aβ plaque accumulation is deleterious for the brain as it causes loss of long term potentiation, synapse damage (which interferes with neuronal cell communication), and cell death. It is also interesting that both 2μM BPA and 20μM BPA exacerbate Aβ toxicity in macrophages, killing many more cells than either BPA or Aβ treatment alone. BPA and Aβ produce greater cytotoxicity, suggesting that BPA can aggravate existing AD. Thus, through its cytotoxicity, BPA plays an essential role in accelerating the AD pathogenesis.

Broadly, BPA is an foreign, environmental pollutant that does not naturally belong in the body or in nature and a disturbance in the microenvironment. Microenvironment disruptions frequently lead to abnormal activation of the immune cells, contributing to the pathogenesis of a variety of inflammatory diseases (Lawrence, 2009). BPA-induced activation of macrophages (via macrophage death) and dose-dependent elevation of IL-1beta and TNF-alpha levels serve as evidence of BPA's role in neuroinflammation in the brain (Li et al., 2018). High IL-1beta levels indicate a pro-inflammatory response, and elevated TNF-alpha levels mean increased activation of the inflammatory response, since TNA-alpha plays a central role in this process (Lawrence, 2009). Elevated pro-inflammatory cytokine levels corroborate past research that aberrant macrophage activation (Li et al., 2018) results from a major loss of these cells and demonstrate that BPA plays a significant role in neuroinflammation. More importantly, neuroinflammation is an AD histological hallmark and essential for the development of neurodegenerative disease.

4.4 BPA's relationship with the estrogen receptor

To elucidate the mechanism by which BPA exerts its deleterious effects, tamoxifen, an estrogen receptor antagonist, and BPA were co-incubated in neuronal cells. From the viability assay, it is apparent that the two chemicals worked synergistically, causing extreme neuronal loss. It is worthwhile noting that this pattern does not apply to the APP ELISA data. There, tamoxifen works against the lower BPA concentration, reducing APP levels rather than increasing it or working synergistically.

Elucidation of BPA's mechanism was performed in the same way for macrophages. The immune macrophages were co-incubated with BPA and 17beta-estradiol (E2), an estrogen receptor agonist. The co-incubation treatment did not produce significantly different survival rates, despite the fact that 20μM BPA by itself was more cytotoxic than 2μM treatment alone. This may suggest that BPA and E2 interact with the estrogen receptor and thus experience competition in receptor binding, which explains the absence of significant difference in survival rates. Moreover, the survival rates are closer in value to that of E2 alone than those of BPA alone, indicating that E2 possibly has greater affinity for the receptor than BPA does, because effects exerted in the combo treatment are similar to those of E2 alone. Similar to neuronal cell data, immune cell data displays synergism between tamoxifen and BPA, but the synergism disappears in ELISA data. Once more, the tamoxifen lowered pro-inflammatory cytokine level of IL-1beta to levels comparable to tamoxifen.

Regardless of increases or decreases in the ELISA assay, BPA-induced elevation in levels of IL-1β elucidate that BPA's specific neurotoxic effects in AD may be exerted through the estrogen receptor, since IL-1β production is regulated by the NF-kB pathway and influenced by estrogen receptor alpha (Shih, Wang, & Yang, 2015). Alzheimer's Disease is known for disproportionately affecting women (Imtiaz et al., 2017; Janicki & Schupf, 2010), and estrogen therapy has shown to significantly alleviate or retard the disease progression under certain circumstances (Imtiaz et al., 2017; Janicki & Schupf, 2010). Indeed, the crucial role of estrogen in AD pathogenesis and BPA's estrogenic activity strongly suggest of BPA's interference and involvement of AD progression.

5. CONCLUSION

From the observations and analysis of data, is it clear that BPA exposure has strong link to Alzheimer's and plays a role in exacerbating the disease. Low survival rates induced by BPA indicate that this xenoestrogen is neurotoxic and can exacerbate present amyloid beta toxicity. Moreover, BPA can induce build up of APP levels with a dose-response effect, which suggest that higher levels of BPA mean greater deposition or formation of amyloid beta plaques, a side effect seen with patients suffering from AD. Furthermore, BPA is cytotoxic to macrophages, which led to abnormal secretion of pro-inflammatory cytokines, IL-1beta and TNF-alpha, and implicates BPA in neuroinflammation, which worsens current AD or may increase risk for AD onset. Lastly, BPA has demonstrated that through its estrogenic activity (as observed with its interactions with E2 and TAX in cell viability assays), BPA can severely interfere with cells in the presence of other substances that interact with the estrogen receptor. In conclusion, BPA produces neurotoxicity and immune cytotoxicity seen in Alzheimer's Disease through estrogenic activity, which urges for the need to expand BPA and Alzheimer's research into animal and human models.

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