Name: Kallista Zhuang
Project Title: Xenoestrogen Bisphenol-A's Neurotoxicity via Estrogenic Activity and Resulting Alzheimer's Disease Pathogenesis
Category: Cellular and Molecular Biology

a. RATIONALE: Include a brief synopsis of the background that supports your research problem and explain why this research is important and if applicable, explain any societal impact of your research.

Bisphenol-A is prevalent in the environment due to its heavy usage in many manufactured products (plastic food containers, beverage containers, toys, medical dental products, etc) and ability to leach at certain temperatures and pH conditions. BPA has potential to disrupt the activity of estrogen receptors and may lead to negative reproductive effects. While there are experiments that have studied BPA's impact on the central nervous system (CNS), there is not a lot of research linking BPA to Alzheimer's Disease (AD), a neurodegenerative disease that is the most common form of dementia. There are some studies, however, that have presented results, demonstrating BPA's ability to interfere with general signaling systems that are important for maintaining cell health, synaptic plasticity, and inflammation: estrogen receptor signaling, NF-kB pathway, Ca2+ flux. This current experiment is examining more closely at these pathways to focus on the BPA's impact and specifically determine whether BPA can induce AD symptoms. This study is also important because plastic is found worldwide and has been key to a fast-paced, industrialized world. Examining closely the effects of BPA may lead to unprecedented benefits for those at risk of AD and encourage the public to reduce its dependency of plastic products.

b. RESEARCH QUESTION(S), HYPOTHESIS(ES), ENGINEERING GOAL(S), EXPECTED OUTCOMES: How is this based on the rationale described above?

Research Question: Is there an increased chance of BPA to induce Alzheimer's Disease or conditions mimicking AD via BPA's interference with the estrogen receptor signaling pathway and resulting cytotoxic effects?

Hypothesis: Due to its ability to bind to estrogen receptors and therefore disrupt estrogen signaling, Bisphenol-A will induce toxic effects and promote inflammation, consequently increasing the chance of the pathophysiological formation of Alzheimer's Disease.

c. Describe the following in detail:

- **Procedures**: Detail all procedures and experimental design including methods for data

collection. Describe only your project. Do not include work done by mentor or others.

- **Cell Culture**
    - Cells to be used in experiment: HTB-11, RAW 264.7
    - Cell Culture maintenance: Incubation at 37℃ at 5% CO2
- **MTT Assay**: a colorimetric assay that measures cell viability/cell survival rate.
  MTT is a tetrazolium dye. In viable cells, NADPH enzymes are able to reduce MTT and form formazan, which has a purple color. Depending on the amount of living cells, the formazan will vary with intensity. The more living cells there are, the more intense the formazan color. The general procedure is below.
    - Put 5 μL of each treatment in the designated wells
    - Incubate at 37℃ and 5% CO2 for 24 hours
    - 10 μL of MTT into each well
        - MTT, or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, is a tetrazolium dye used in the MTT colorimetric assay, which will be used to assess cell metabolic activity. NAD(P)H-dependent cellular oxidoreductase enzymes in viable cells reduce the MTT into the dye's insoluble formazan. MTT assay can also be used to measure cytotoxicity (loss of viable cells). Incubation of cells with MTT reduces the formazan product (the enzymes still reduce MTT, but incubation prevents insoluble formazan from interfering with the optical density).
        - DMSO is a powerful solvent that can dissolve the MTT formazan crystals
    - Incubate for 2 hours
    - Add DMSO to each well
        - The DMSO
    - Let sit in incubator for 10-15 minutes
    - Read using the cell plate reader at a wavelength of 595 nm, the top left corner in the reader is A1 (where the missing corner piece of the cell plate is)
    - Data Collection: Analyze the data in excel; calculate the average and standard deviation for each treatment type. Using control, determine the cell viability rate for each treatment. Create a graph with viability rates. Use ttests to determine significance.

For the placement of treatments in each well, the number of treatments per well can range from 1 to 4. Testing one chemical in a well is used as a control for the combination groups. The different combination groups are to assess whether the treatments are able to interact with one another. For example, two of the chemicals used in this experiment so far are BPA and tamoxifen. Tamoxifen is an estrogen modulator and can be used to treat breast cancer. Like tamoxifen, BPA has partial agonist/antagonist effects of the estrogen receptor, depending on BPA concentration. To add to the complexity, BPA can exert different agonist/antagonist effects depending on the cell type. Thus, to examine whether BPA and tamoxifen interact in the cell lines, one column

will have only BPA treatment, the next column was only treated with tamoxifen, and a third column had wells with both BPA and tamoxifen. After reading the plate at 595 nm, I will compare the absorbance of the treatment relative to that of control. I will then generate the cell viability rate.

- **LDH Cytotoxicity Assay**: measures the cytotoxicity/toxicity of a chemical to a cell
    - Put 5 µL of treatment in designated wells
    - Incubate at 37℃ and 5% CO2 for 24 hours
    - Defrost the lysis and LDH substrate
    - Take 30 µL of cell media out of the original and put into a new plate
    - Put 10 µL lysis in each well of the original plate → incubate for 45 mins
    - Put 30 µL of LDH substrate in each well of the new plate → leave **in a dark area for 30 minutes**
    - Put 30 uL of LDH stop solution
    - Read using the cell plate reader at a wavelength of 490 nm
    - Pop the bubbles
    - Put the plate in the dark, take 30 uL out and put in a new plate. Add 30 uL LDH. Wait for 20-30 minutes.
- **Cell Migration Assay**: (put the full assay in here)
- **Enzyme-Linked Immunosorbent Assay (ELISA)** will be used to determine cytokine expression

To study the protein expression of cells, this project will use 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 that will be obtained from treatment will be compared to the standards. In preparing the samples, the cell culture will be cleared of media and then will be stored at -20 degrees Celsius. Before using, bring all the reagents to 37 degrees. The wash powder will then be dissolved in 1000mL of water to create the 1X PBS wash buffer.

For Biotinylated Anti-Human IL1B antibody reagent, this reagent will be prepared immediately prior to use through a dilution of 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 will 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. Remove excess microplate strips from the plate frame and seal and store them in the original packaging.
2. 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).
3. Cover with the plate sealer provided and incubate for 120 minutes at RT (or 90 min. At 37 degrees C).
4. 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.
5. Add 100 microliters of the prepared 1x Biotinylated Anti-Human IL1B antibody to each well.
6. Cover with plate sealer and incubate for 90 minutes at RT (or 60 minutes at 37 degrees C).
7. Wash the plate 3 times with the 1x wash buffer.
8. 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)
9. 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.
c. Repeat steps a-b 4 additional times.

10. 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).
11. Add 100 microliters of Stop Solution to each well. The color should immediately change to yellow.
12. Within 30 minutes of stopping the reaction, the O.D. absorbance should be read with a microplate reader at 450 nm.

• **Risk and Safety**: Identify any potential risks and safety precautions needed.

All cell lines are BSL-1. Cell treatments are used on micromolar level (Note: cell wells themselves have liquid media, so concentrations handled will be different from the concentrations the cells experience).
Chemical (Concentration to be handled)

of BPA, tamoxifen, OKA, LPS, estradiol, amyloid-beta peptide). Cells will be disposed in their plates in a biohazard waste bin after assay. Chemicals are expected to be used completely. If not, they can be used for future research or disposed of in a biohazard waste bag.

When handling chemicals or cell lines, gloves, goggles, and a coat will be worn at all times as some chemicals used may cause skin irritation. Almost all procedures will take place under the hood. Furthermore, after each use, the hood must be cleaned thoroughly with ethanol. After using and cleaning the hood, germicidal UV will be turned on.

• **Data Analysis:** Describe the procedures you will use to analyze the data/results.

ANOVA will be used to analyze significant differences between treatment groups. Graphs will be grouped together based on their treatment type and cell type for clarity.

d. BIBLIOGRAPHY: List major references (e.g. science journal articles, books, internet sites) from your literature review. If you plan to use vertebrate animals, one of these references must be an animal care reference.

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Addendum:

- Over the course of my project, I settled the project's focus to two different mechanisms of inducing Alzheimer's Disease: through neurotoxicity and inflammation.
- Instead of using ELISA only for measuring cytokine protein levels in the cell wells, I decided to use the assay for measuring another Amyloid Precursor Protein (APP) levels to directly measure how BPA is influencing proteins directly related to AD.
- I decided to not use the cell migration assay because of the focus on investigating in depth two mechanisms.