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Cellular and Molecular Biology
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Ecotoxicity of Bisphenol S Through Proteomic and Genomic Changes in *C. elegans*: Preliminary Results

Rationale

It is estimated that 6.3 billion tons of plastic have been disposed of into water[1]. Bisphenol A (BPA), an important precursor for synthesis of polycarbonate plastics and epoxy resins, has been found to be in 2.2 million tons of plastic in 2009[2,3]. However, a number of negative correlations with BPA has caused industry to shift towards Bisphenol S (BPS), an analog of BPA[4]. Now present in baby bottles, thermal receipt papers, food packaging materials, and personal care items, it is increasingly disposed into the environment through both industrial and personal object disposal. Furthermore, BPS has been determined to be in low, but noticeable amounts in bodies of water in Asia[5]. BPS therefore will affect both humans and the ecological systems that it is contaminating. Now ubiquitous, it is critical that BPS is well-characterized in order to screen for potential effects on organisms as exposure and accumulation of the chemical increases.

Toxicity screening has typically been performed *in vitro* through tissue culture and *in vivo* using simple mammalian systems. However, *in vitro* remains unpredictable at the organismal level, and immortalized cell lines may trigger false-positives and false-negatives.[6] Mammalian models are both expensive, time-consuming, and rodent models are only predictive for humans about 50% of the time.

Caenorhabditis elegans is an alternative model for toxicity screening. The small worm boasts many advantages, from its large brood size and transparent body to ease of genetic manipulation and well-characterized genome. *C. elegans* has already been a critical model organism for elucidating numerous behavioral and molecular mechanisms, including the seminal discoveries in apoptosis, siRNA, and aging. Many of the features that made *C. elegans* a prime animal model in the fields it is well known for, from neurobiology, developmental biology, and genetics, the same characteristic make it a prime candidate for toxicity screening.[6]

Because *C. elegans* is so prevalent in soil, aquatic and sediment environments, the nematode can play a pivotal role in providing whole-organismal data for ecotoxicology. Mounting evidence suggests that its genetic and physiological traits are able to result in predictive toxicity data for higher eukaryotes.[7] Many physiological processes within the worm are observed in higher organisms, humans included. Indeed, LC50 rankings in *C. elegans* have been as predictive of acute toxicity in mammals as rats. They particularly will be useful for this study: endocrine disruption and mitochondrial DNA mutations.

BPS's better known cousin BPA is known to have an endocrine-disrupting capacity due to its structural features, acting as an agonist and agonist for different members of the estrogen

receptor family.[8,9] Nuclear hormone receptors (NHRs) are group of transcriptional regulators easily identifiable through two traits, a well-conserved DNA binding domain consisting of two zinc fingers and a ligand-bonding domain at the C-terminal.[10,11] These proteins are involved in a slew of cellular and organismal processes.[12]

While *C. elegans* has a dramatically larger number of NRs than humans (284 to about 50), the *C. elegans* gene NHR-14 is homologous to the human estrogen receptor ERα, as evidenced by sequence similarity and confirmed binding with natural ligands.[12] It is therefore reasonable to think that BPS may display many of the same characteristics and may act as an agonist or antagonist for NHR-14 and thereby its homologous counterparts in other organisms. It is sensible to detect whether BPS is indeed causing endocrine disruption before proceeding onto other effects it may be causing on the worms.

While mitochondria are most known for generation of ATP and apoptotic induction through oxidative phosphorylation, they also have their own homeostatic processes such as mitochondrial DNA (mtDNA) replication, mitochondrial biogenesis, and mitochondrial fusion and fission. Mitochondria and mtDNA furthermore may be particularly susceptible to environmental stressors and lack some repair mechanisms present in the nucleus. Furthermore, the compact sequence, lacking new introns and non-coding regions, means that any damage to the DNA may be even more impactful upon the organelle. mtDNA integrity and general mitochondrial function is therefore important for organisms. Investigating mtDNA damage therefore may serve as a particularly sensitive biomarker for ecotoxicity.

Research Questions

- Does BPS induce proteomic and genomic changes?
- Can Type I NADH dehydrogenase respiratory complex I be used as a marker for mtDNA damage?

Specific Aims

- Determine LC50 doses of BPS for *C. elegans* and establish acute toxicity dosage concentration and interval
- Determine whether BPS is indeed an endocrine disruptor in *C. elegans*
- Investigate correlations between mutations in the sequence encoding for mitochondrial complex and mitochondrial homeostasis

Hypothesis

- Acute BPS exposure will induce mutations in *C. elegans* mtDNA, thereby disturbing the worm's metabolism, ATP levels and normal ratio of NAD/NADH
- BPS interacts with NHR-14 receptors, therefore impacting the brood sizes and maturation rates

Expected outcomes:

BPS is expected to induce mutations in mtDNA and for disruption of mitochondrial homeostasis to be further evidenced by lower levels of ATP and changes in the NAD+/NADH ratio. Furthermore, if BPS exhibits similar endocrine disrupting abilities as BPA, BPS is posited to be

found interacting with NHR-14 as either an agonist or antagonist. Smaller or nonexistent broods and retarded maturation can be expected to indicate a strong correlation between BPS and endocrine disruption.

Methods and analysis

Treatment

BPS in absolute ethanol will be diluted at 0, .1, 1, and 10 mM in NGM agar. 1 ml of treatment agar will be pipetted into each well of a 12-well plate. After inoculating plates with OP50, worms synchronized to the L1 stage will be picked onto the plate for appropriate exposure times.

Worm Viability Assay

Treated worms will be collected in 100 µl of M9 wash buffer and suspended homogeneously in the solution. 10 µl of the subsequent solution will be pipetted onto a glass slide. Thrashing worms will be considered live.

Brood size and maturation rate

While an equal number of worms will be treated per experimental group, the ratio of mature *C. elegans* to eggs on the plate will determine brood size. Similarly, the number of mature to juvenile, L1-L3, worms will be counted in order to investigate if BPS potentially retards growth.

ATP Luciferase Assay

Levels of ATP are an indicator for general organismal health (and therefore can be used to titer BPS) and for potentially metabolic disturbances. Worm lysates will first be normalized using a BCA assay followed by analysis by BioVision's ATP Colorimetric Assay Kit, which relies on the phosphorylation of glycerol to give a reading of ATP concentrations.

Colorimetric NAD+/NADH Quantification

Like ATP levels, the ratio of NAD+ to NADH can help titer BPS and indicate metabolic disturbances. BioVision's colorimetric ELISA will be performed on age-synchronized *C. elegans* to quantitatively compare NAD+/NADH ratios.

Determining mutagenesis

DNA will be isolated using Nemametrix' *C. elegans* Worm Lysis Kit. Each worm will be picked into a mixture of the kit's solutions followed by a fifteen minute boil. After a ten-fold dilution, DNA extracts will amplified via PCR (using reagents from Nemametrix' PCR Master Mix) before being sent out for sequencing. Primer design was taken from Claudia P. Gonzalez-Hunt et al.[13]

Forward primer sequence: 5′-CAC ACC GGT GAG GTC TTT GGT TC-3′

Reverse primer sequence: 5′-TGT CCT CAA GGC TAC CAC CTT CTT CA-3′

Co-Immunoprecipitation (Co-IP)

Co-IP will be used to isolate the proteins BPS is interacting with. The worm protein lysate will be incubated with anti-BPS antibodies, which will attach to the BPS in the BPS-protein complex. The immune complex will be precipitated by beads with antibody-binding protein and subsequently centrifuged to pull the beads down. Because the beads isolate the BPS and its interacting proteins, any other proteins not interacting with BPS can be washed away.

Western Blotting

After boiling the isolated protein complex to denature the proteins, the pulled-down proteins from the co-IP will be run through a polyacrylamide gel to separate the samples. The proteins will then be eluted, the membrane blocked, and BPS and NHR-14 targeted through their respective anti-BPS and anti-NHR-14 antibodies before staining with a secondary antibody to visualize.

Hazardous Chemicals

1. **List all hazardous chemicals, activities, or devices that will be used; identify microorganisms exempt from pre-approval:**
   This team will utilize Bisphenol S at a concentration of 10mM. All stock and working dilutions will be prepared for student use by the mentor/teacher (Ileana Rios).

2. **Identify and assess the risks involved in this project:**
   Due to stringent safety protocols, safety training, and direct supervision, the risks to the students are minimal and may involve accidental spills.

3. **Describe the safety precautions and procedures that will be used to reduce the risks.**
   Skin contact and eye exposure are entirely minimal due to personal protective equipment; In addition, there is a shower and eye wash station in the biology lab. First, the BSL-1 prep room which houses the CO_2 incubator, cell media reagents, autoclave, and biohazardous waste is secure with a combination door lock; a few instructors in the science department and maintenance are familiar with the key code; the prep room is always closed and locked unless Dr. Rios is present in the room. Students will wear personal protective equipment consisting of lab coats, nitrile disposable gloves, goggles, and facemasks from VWR. All activities and protocols with BSL-1 entities are carried out in the safety hood in a BSL-1 prep room and under direct supervision by Dr. Rios. All stock and working dilutions of Bisphenol S will be prepared by Dr. Rios for student use.

4. **Describe the disposal procedures that will be used (when applicable)**
   All worm media waste is disinfected with 10% bleach and autoclaved for 20 minutes at 212^{o}F prior to disposable in a red biohazard bag which is picked up for incineration by *Sharps* Compliance, Inc. **All Bisphenol S-treated liquid waste is collected in amber chemical waste bottles and collected by PEGEX Hazardous Waste Removal (Account Number A-96207).**

   List the source(s) of safety information:
   a. Sigma Aldrich Safety Data Sheet

b. Product Name: Bisphenol S
c. Product Number: 43034
d. Brand: Sigma Aldrich
e. CAS Number: 80-09-1

Citations

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7. Hunt, Piper Reid. "The C. elegans model in toxicity testing." Journal of applied toxicology : JAT vol. 37,1 (2017): 50-59. doi:10.1002/jat.3357
8. Schug, Thaddeus T., et al. "Minireview: Endocrine Disruptors: Past Lessons and Future Directions." *Molecular Endocrinology (Baltimore, Md.)*, vol. 30, no. 8, Aug. 2016, pp. 833–47, doi:10.1210/me.2016-1096. PubMed, 27477640.
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11. Magner, Daniel B., and Adam Antebi. "Caenorhabditis Elegans Nuclear Receptors: Insights into Life Traits." *Trends in Endocrinology and Metabolism: TEM*, vol. 19, no. 5, July 2008, pp. 153–60, doi:10.1016/j.tem.2008.02.005. PubMed, 18406164.

12. Antebi, A. Nuclear hormone receptors in C. elegans (January 03, 2006), WormBook, ed. The C. elegans Research Community, WormBook, doi/10.1895/wormbook.1.64.1
13. Gonzalez-Hunt, Claudia P., et al. “PCR-Based Analysis of Mitochondrial DNA Copy Number, Mitochondrial DNA Damage, and Nuclear DNA Damage.” *Current Protocols in Toxicology*, vol. 67, no. 1, 2016, pp. 20.11.1-20.11.25, doi:10.1002/0471140856.tx2011s67.