Effects of Chronic and Acute Bisphenol-A Exposure On the Cellular Stress Response

A Major Qualifying Project Report

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ABSTRACT

Stress granules (SGs) are cytoplasmic aggregates of mRNA and protein that form in response to environmental stress and are thought to confer protection from apoptosis. Bisphenol-A (BPA) is a ubiquitous environmental toxin linked to numerous diseases, though its relationship to SGs was previously unknown. BPA exposures within human serum levels had no significant effect on cell growth nor induced SG formation. However, high-dose acute BPA exposure greatly induced SG formation. Further, low-dose chronic BPA exposure resulted in a significant decrease in SG formation during subsequent high-dose acute BPA exposure, suggesting that chronic exposure to BPA alters cellular stress dynamics. Our results suggest that BPA may seriously alter the ability of cells to cope with environmental stress.

ACKNOWLEDGEMENTS

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BACKGROUND

Cellular Stress Response

During environmental stress, eukaryotic cells reprogram their translational machinery to allow the selective expression of proteins required for viability in the presence of the stressor, while simultaneously redirecting housekeeping proteins (Kedersha et al., 2005c). These housekeeping proteins become non-translating mRNAs during the stress, and they can be segregated into two types of cytoplasmic mRNP granules: Processing ("P") bodies that generally contain mRNA decay machinery and stress granules, which contain numerous translational components (Anderson and Kedersha, 2006;Buchan and Parker, 2009). A figure showing an example of stress granules and processing bodies that formed in arsenite treated human U2OS can be seen below in ***Figure 1*** (Anderson and Kedersha, 2009).

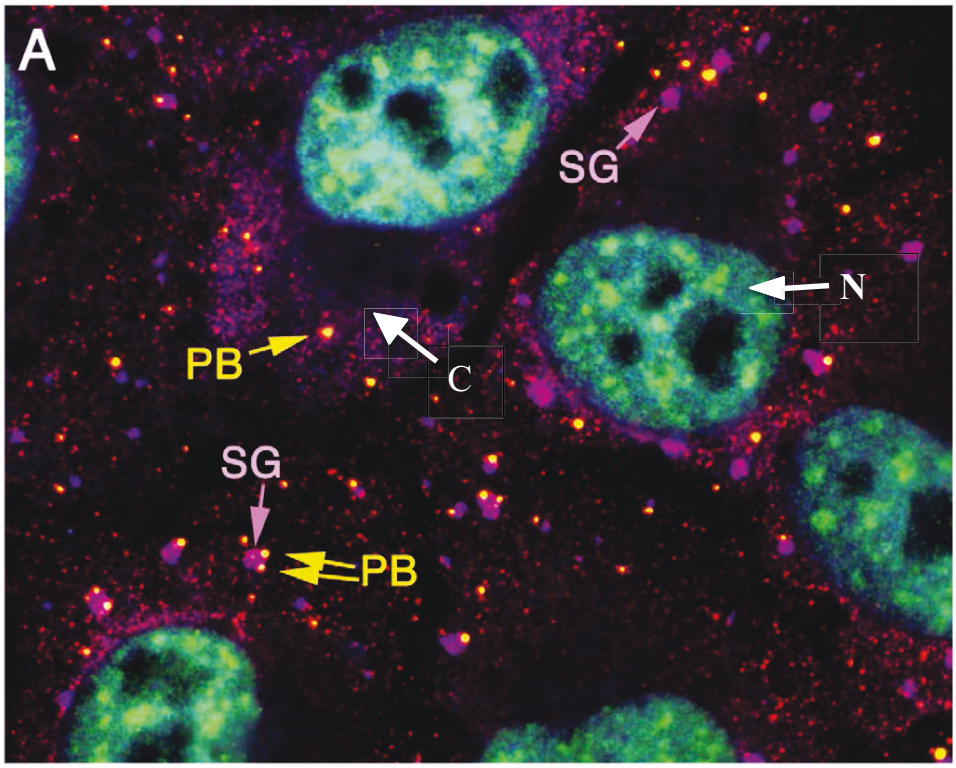

Figure 1: Stress Granules and Processing Bodies in Arsenite Treated U2OS Cells. The stress granules are labeled SG (purple) while the processing bodies are labeled PB (yellow). Stress granules and processing bodies are both cytoplasmic granules. One cellular nucleus is labeled N and appears green while the cytoplasm is labeled C and appears purple. (Anderson and Kedersha, 2009)

Stress Granules

Nover et al first discovered stress granules in heat shocked tomato cells in 1988 (Nover et al., 1989). Stress granules are cytoplasmic RNA-protein complexes containing non-translating mRNAs, translation initiation components, and many additional proteins affecting mRNA function that occur when a eukaryotic cell undergoes stress (Buchan and Parker, 2009). Stress granules are sites of mRNA triage at which mRNP complexes are monitored for integrity and composition and are then routed to sites of re-initiation, degradation, or storage (Kedersha and Anderson, 2002). The formation of stress granules allows the cell to focus its energy and resources on translating proteins necessary for survival by silencing translation of non-essential proteins. An example of this could be the increased translation of heat-shock proteins during an acute exposure to high temperature. These heat-shock proteins would prevent the denaturing of other proteins within the cell increasing the cell's likelihood for survival.

Composition of Stress Granules

Stress granules are primarily composed of 48s pre-initiation complexes: such as, eIF3, eIF4E, and eIF4G. These complexes contain bound mRNA which are derived from disassembling polysomes. ***Figure 2*** below diagrams the stress granule formation cycle as well as the relationship between stress granules and processing bodies (Kedersha et al., 2005c). The components that make up stress granules include poly (A)+ RNA bound to early initiation factors, which regulate translation initiation on stress granule-associated transcripts (Anderson & Kedersha 2006). In an experiment with CV-1 cells treated with sodium arsenite, it was observed that the different components of the stress granule were equally distributed through the cytoplasm and nucleoplasm prior to the sodium arsenite treatment. Once under an acute stress, caused by sodium arsenite, the microtubules within the cell maneuver stress granule components together resulting in the initiation of stress granule formation. The experimental evidence presented shows nocodazole treatment disrupts microtubule formation which then prevents stress granule formation at the specific stress site (Ivanov et al., 2003).

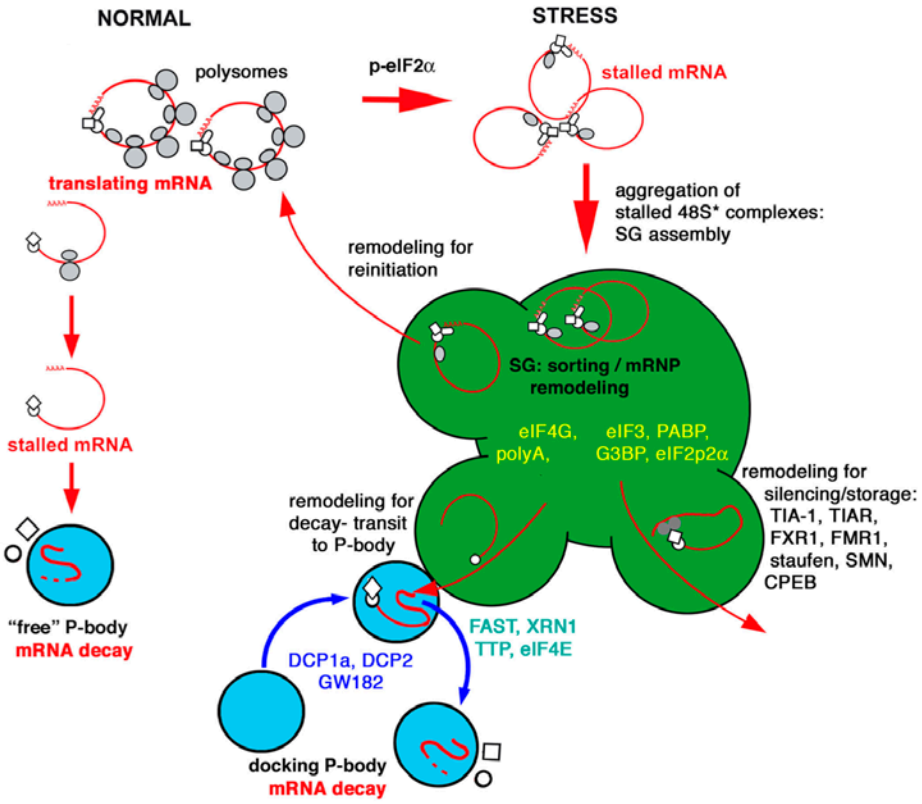

Figure 2: Stress Granule Formation Cycle (Kedersha et al., 2005c)

Functional Mechanism of Stress Granules

When the cell is under environmental stress, such as arsenite treatment, it will exhibit phosphorylation of the eIF2(alpha) complex. This will arrest protein translation by lowering the availability of the ternary complex, eIF2-GTP-tRNAiMet, thus leading to an increase in untranslated mRNA. These untranslated mRNAs are then processed by stress granules (Ivanov et al., 2003). It is suggested that the primary functions of stress granules include the degradation, storage, and translation of mRNA. It is proposed that stress granules form to regulate translation in cells that are under stressed conditions in order to up-regulate the translation of proteins necessary for survival while simultaneously down-regulating the translation of housekeeping and non-essential mRNAs (Kedersha et al., 2002).

Arsenic Induction of Stress Granules

Arsenic is an element in group V of the periodic table of elements. It has five valence electrons which results in it being highly reactive. In the atmosphere, arsenic acts as an oxidizing agent when heated. Initially arsenic was used for applications such as

wood preservation because it was found to be an effective insecticide. Eventually, it was realized that arsenic was more toxic than initially thought. Chronic exposure to inorganic arsenic has been linked to skin, lung and bladder cancer. There has also been evidence that arsenic exposure may lead to liver and prostate cancer, diabetes, cardiac disorders, and neurological problems. Studies have shown a strong correlation between arsenic exposure and cancer incidence (Hong et al., 2014).

With this view of arsenic as a toxin, it has been used commercially for applications like rat poisons and insecticides. Many researchers have looked into the impact arsenic has at a cellular level. Bhattacharya and Bhattacharya studied the effect of arsenic in nonlethal doses on *Clarias batrachus* (Indian catfish) and observed the impact on cellular processes by measuring the change in the level of byproducts. They found that a nonlethal dose of arsenic "induced tissue lipid peroxidation, increased the ratio of oxidized to reduced glutathione and produced excess H_2O_2 within 1–2 days of exposure" (Bhattacharya and Bhattacharya, 2007). Their study also showed arsenic treatment to impact the activity of glutathione peroxidase, superoxide dismutase, catalase, and glutathione reductase. The resulting fluctuations in these enzyme levels indicates oxidative stress. This was found to be reversible by pretreatment with N-acetylcysteine which could indicate the oxidative stress is caused by the excess H_2O_2 production (Bhattacharya and Bhattacharya, 2007).

Oxidative stress interferes with the normal redox state of the cell. This includes the level of redox intermediates as well as enzymes involved with redox reactions. When a cell is experiencing oxidative stress they produce excess peroxides which they cannot readily break down. These peroxides damage cellular components such as proteins and DNA. The resulting DNA damage, if not repaired immediately, can lead to apoptosis or cellular transformation. An imbalance of the redox state of a cell will also interfere with signal transduction. The interference with cell signaling can disrupt many processes within the cell. Oxidative stress has been hypothesized to have involvement in the development of various diseases including cancer (Suzuki et al., 2008).

Various studies have shown that arsenic induces cytoplasmic granules, for example ***Figure 3*** shows the formation of stress granules in U2OS cells after arsenite treatment (Kedersha et al., 2005). The stress granules are forming in response to the

oxidative stress caused by arsenic. Since the stress caused by arsenic has been shown to be measurable in these two different ways (quantification of oxidation related molecule levels and stress granule formation) it may be possible to quantify the stress caused by other environmental toxins by these two methods (Jacobson et al., 2012).

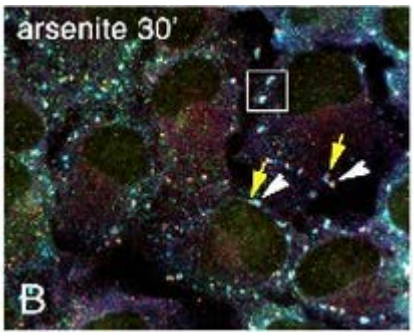

Figure 3: Stress Granule Formation After 30 Minute Arsenite Treatment
(Kedersha et al., 2005)

Bisphenol-A

Bisphenol-A (BPA), 2,2-bis(4-hydroxyphenyl)propane, is a chemical made by combining acetone and phenol that is used primarily to produce polycarbonate plastics and epoxy resins (Kang et al., 2006). Polycarbonate plastics are utilized in the production of baby bottles, reusable plastic bottles, plates, cups, microwave ovenware, and storage containers. Epoxy resins are used for the internal coating of food and beverage cans (Geens et al., 2011). The prevalence of BPA in food storage containers contaminates the food, which leads to the unintentional human consumption of BPA. BPA is also found in thermal printed-paper, which is typically used in sales receipts, meaning that skin is another avenue of human exposure to BPA (Biedermann et al., 2010). BPA is one of the highest volume chemicals produced worldwide -- an estimated 6 billion pounds are produced annually-- and this production releases over 100 tons into the atmosphere (Vandenberg et al., 2007). BPA has been found in bodies of water, soil, and the air surrounding factories and hazardous waste landfills (Kang et al., 2006).

Human BPA Exposure Levels

Numerous studies have been conducted in order to quantify the average level of exposure of humans to BPA. Studies have revealed that BPA can be measured in the serum, urine, amniotic fluid, follicular fluid, placental tissue, and umbilical cord blood of humans, most likely as a result of exposure to BPA through the aforementioned products

and environmental exposures(Vandenberg et al., 2007). The average level of BPA in healthy human serum, as determined by a 2000 study, is 1.40nM (Inoue et al., 2000). Numerous studies have found that the daily human intake of BPA is <1µg/kg body weight/day (Bolt et al., 2001). Although this is considerably lower than the no-observed-adverse-effect level (NOAEL) of 5 mg/kg body weight/day, concern has been raised by several studies that suggests levels as low as about 0.21µg/kg body weight/day can cause health problems (Kang et al., 2006;Vandenberg et al., 2007).

Deleterious Effects of BPA

The levels of BPA found in human tissue have raised concern because BPA is considered an endocrine disrupting chemical. Endocrine disrupting chemicals interfere with the endocrine system, which is a collection of glands that secrete hormones to regulate various body functions such as reproduction, growth and development, maintenance of electrolytes, water, and nutrient balance of the blood, regulation of cellular metabolism and energy balance, and mobilization of body defense (Marieb & Hoehn, 2012).

BPA mimics the hormone estrogen in humans and has a weak affinity for binding to estrogen receptors (Vandenberg et al., 2007). The estrogen mimicking property of BPA was discovered accidentally by Krishnan et al. when BPA leached from autoclaved polycarbonate flasks increased the rate of proliferation of MCF-7 breast cancer cells (Krishnan et al., 1993). Further evidence of BPA's affinity for binding to estrogen receptors can be seen when comparing the chemical structure of BPA to diethylstilbestrol (DES), which was a synthetic form of estrogen prescribed to pregnant woman between 1940 and 1971 that was later found to cause cancer, birth defects, and other developmental abnormalities (Giusti et al., 1995). As shown below in ***Figure 4***, BPA has a similar structure to DES, and although it is different from that of endogenous estradiol, it is still capable of binding both alpha and beta estrogen receptors (Vandenberg et al., 2007).

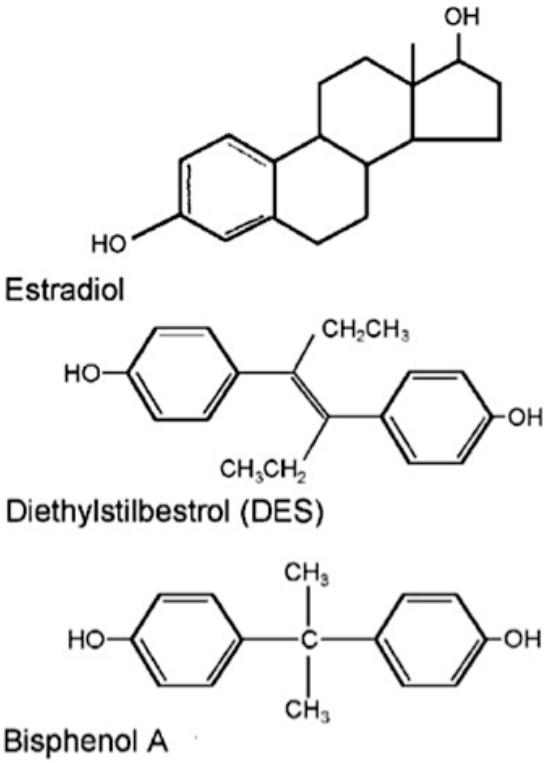

Figure 4: Chemical Structures of Estradiol, BPA, and DES
(Vandenberg et al., 2007)

Due to its estrogen mimicking capabilities, BPA has been linked to numerous chronic diseases, such as diabetes, cardiovascular disease, chronic kidney disease, birth defects and developmental disorders, reproductive disorders, cancer, and chronic respiratory disease (Rezg et al., 2014).The exact relationship between BPA exposure and these chronic diseases as well as the mechanisms has not been established, but epidemiological studies have shown a positive correlation.

BPA and the Cellular Stress Response

BPA has been shown to impact the levels of superoxide dismutase, catalase, glutathione reductase, and glutathione peroxidase. All of these enzymes are involved with the cells oxidative stress response. If BPA is interfering with the cellular redox state as severely as arsenic it is very likely that BPA would also cause the formation of stress granules. If BPA is causing the same cellular stresses as arsenic, it is important to know because there are known methods, such as N-acetylcysteine treatment, for reducing this cellular stress (Chitra et al., 2003).

In the context of our project, we aim to characterize the acute and adaptive cellular stress response to BPA exposure. We plan on accomplishing this through completing the following objectives:

1.) *To test if BPA levels similar to average human serum levels have an impact on cellular growth and stress granule formation.*

2.) *To assess the impact of acute high-level BPA exposure on stress granule formation.*

3.) *To analyze the impact of a chronic low-level BPA exposure on SG formation during a subsequent acute high-level BPA exposure, also known as an adaptive response.*

In conjunction with these objectives, we hypothesized that the following outcomes would occur:

1.) *Chronic low-dose exposure to BPA will result in a decrease in cellular growth that is dose dependent.*

2.) *Acute high-dose exposure to BPA will lead to the formation of stress granules.*

3.) *Chronic low-dose exposure to BPA prior to an acute high-dose BPA exposure will decrease the amount of stress granule formation, also known as an adaptive response.*

This foundational knowledge could provide significant implications for the connection between BPA exposure and cellular apoptosis as well as BPA related disease states. Establishing this connection could raise awareness and help improve prevention practices for BPA exposure.

METHODS

U2OS Cell Line

U2OS is a human osteosarcoma cell line that was first cultured in 1964 from the tibia of a 15 year old female (Niforou et al., 2008). This adherent U2OS cell line was transfected with GFP-G3BP and then cloned (Kedersha et. al, 2008). G3BP is a phosphorylation-dependent endoribonuclease that localizes mRNA and regulates stress granule assembly (Tourrière et al., 2003). The GFP florescent tag helps to visualize the localization of G3BP for the identification of stress granule formation through microscopy. The U2OS GFP-G3BP cell line was a kind gift from Nancy Kedersha at Brigham and Women's Hospital in Boston. This cell line is cultured in DMEM (Corning Cellgro) media with 10% FBS (Equitech) and 1% Penicillin/Streptomycin (Lonza) and incubated at 37°C with 5% CO_2. The cultures are allowed to grow for 2-3 days then subcultured 1:3.

BPA Survival Assay

1.0×10^5 U2OS cells were plated in each well of a six-well plate and grown for 48 hours. A specific concentration of BPA (Aldrich Chemistry) was introduced to each well and the cells grew for an additional 24 hours in the incubator. The following concentrations of BPA diluted in cultured media were tested: 1.09nM, 2.19nM, 4.38nM, 8.75 nM, 17.5nM. (The initial BPA stock used was dissolved in methanol at a diluted concentration of 0.1M.) At 24 hours, cells were treated with Trypsin-Versene, scraped, collected into an Eppendorf tube, dyed with 0.4% trypan blue and then the live cells were counted using a hemocytometer. To analyze the statistical significance of the data from this experiment we performed an ANOVA using Microsoft Excel.

Acute Stress Response

To induce stress granule formation in the U2OS cell line, various acute stress tests were performed. 7.0×10^4 U2OS cells were plated in each well of a twelve well plate on top of a coverslip that had been pretreated with 125µL of DMEM 1X+20% FBS+Pen/Strep media for 5 minutes. The cells grew for 48 hours in 1 mL of DMEM 1X+10% FBS+Pen/Strep media in a 37ºC incubator. After this period of normal growth, a specific concentration of the stressor [either sodium arsenite (Aldrich Chemistry) or BPA (Aldrich Chemistry)] was introduced to each well and incubated for 1 hour. The

following concentration of sodium arsenite diluted in cultured media was utilized: 500µM. The following concentrations of BPA diluted in cultured media were utilized: 500µM, 400µM, 300µM, and 250µM. At the end of the 1-hour treatment, the treated media was aspirated off and the cover slips were covered in 1X PBS. The cells in each well were then fixed with paraformaldehyde, treated with 100% methanol, and the nuclei were subsequently stained with Hoechst 33342 (Life Technologies). The cover slips from each well were then mounted, cell side down, on labeled microscope slides utilizing polyvinyvl alcohol mounting media (Fukui et al., 1987).

To quantify the formation of stress granules, a minimum of 100 cells were counted, and classified as either positive or negative for visible stress granules granule formation at 400X magnification on the Zeiss AXIO observer A1. Once a field of view was started, all the cells within it were counted. All images were captured using the SPOT Idea 5.0mp camera attached to the Zeiss AXIO observer A1 microscope. The images were taken using GFP and DAPI channels. The merges for our fluorescence microscopy images were made by overlaying the images obtained from these two channels using Adobe Photoshop. To analyze the statistical significance of the data from this experiment we performed an ANOVA as well as two-tailed T tests with 95% confidence for differences between groups using Microsoft Excel.

Adaptive Stress Response

To test if exposure to a low BPA concentration makes an impact on stress granule formation in a later acute BPA exposure, an adaptive stress response experiment was performed. 7.0 X 10^4 U2OS-GFP-G3BP cells were plated in each well of a twelve-well plate on top of a coverslip, which had been pretreated with 125µL of DMEM 1X+20% FBS + Pen/Strep Media for five minutes. The cells grew for 24 hours in 1 mL of DMEM 1X + 10% FBS+Pen/Strep media in a 37˚C incubator. Wells were then treated according to the chart in ***Table 1*** (all BPA dilutions are in cultured media). The BPA treatments added at 24 hours stayed on the wells the remainder of the experiment while those added at 48 hours lasted 1 hour. The cells in each well were fixed with paraformaldehyde, then with 100% methanol and subsequently the nuclei were stained with Hoechst 33342 (Life Technologies). Each cover slip from the wells were mounted, cell-side down, onto a labeled microscope slide with polyvinyl alcohol mounting media (Fukui et al., 1987).

To quantify the formation of stress granules, a minimum of 100 cells were counted and classified as either positive or negative for stress granules at 400X magnification on the Zeiss AXIO observer A1. Once a field of view was started, all the cells within it were counted. All images were captured using the SPOT Idea 5.0mp camera attached to the Zeiss AXIO observer A1 microscope. The images were taken using GFP and DAPI channels. The merges for our fluorescence microscopy images were made by overlaying the images obtained from these two channels using Adobe Photoshop. To analyze the statistical significance of the data from this experiment we performed an ANOVA as well as a one-tailed T test with 95% confidence using Microsoft Excel.

RESULTS

Sodium Arsenite Acute Stress Response

To ensure our U2OS GFP-G3BP cell line enabled us to visualize stress granule formation, the cells were grown on coverslips in a 12 well plate for 48 hours and then treated with 500µM sodium arsenite for 1 hour, which is a condition that has previously been shown to form stress granules (Kedersha et al., 2005a). After the 1-hour exposure, the cells were fixed, the nuclei were stained with Hoechst 33342, and coverslips were mounted on slides using polyvinvyl mounting media. ***Figure 5*** shows the sodium arsenite treated cells at 630X total magnification.

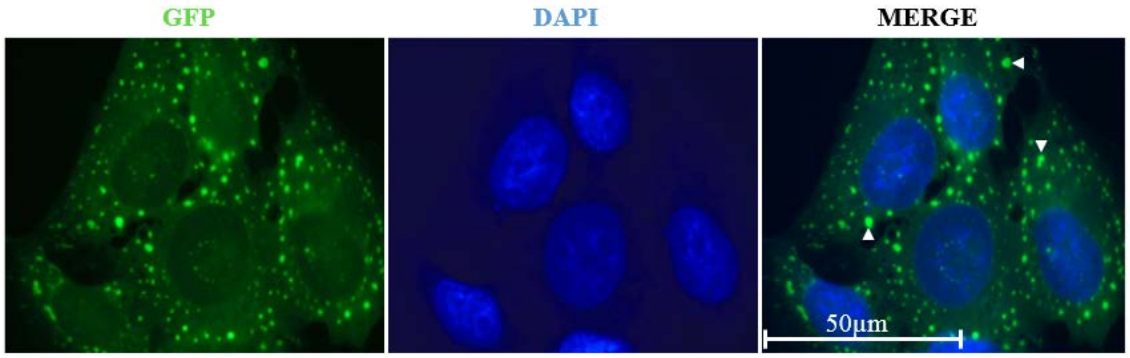

Figure 5: 630X total magnification of U20S GFP-G3BP cell(s) after 1 hour exposure to 500µM sodium arsenite. White Arrows indicate the site of stress granules.

As shown in ***Figure 5***, the 1-hour exposure to 500µM sodium arsenite did in fact induce stress granule formation in 92% of U2OS GFP-G3BP cells, as reported previously.

BPA Survival Assay

In order to test the effect of varying concentrations of BPA on U2OS cell growth, 1.0×10^5 U2OS GFP-G3BP cells were plated in each well of a six well plate. After 48 hours of normal growth, wells were treated with the following concentrations of BPA: 1.09 nM, 2.19nM, 4.38nM, 8.75nM, 17.5nM and the last well was left untreated to act as a control. After a 24-hour exposure, each well was trypsinized and cell counts were performed to quantify the number of remaining live cells. Counts revealed that all 6 wells underwent cell growth and ended with more cells than had been originally plated, which can be seen in Supplemental Data (***Tables S1)***. The cell counts were normalized to the cell growth of the untreated well within each trial. These normalized values from each of

the five trials were averaged. The mean values of normalized cell counts were graphed with standard error bars ***(Figure 6)***.

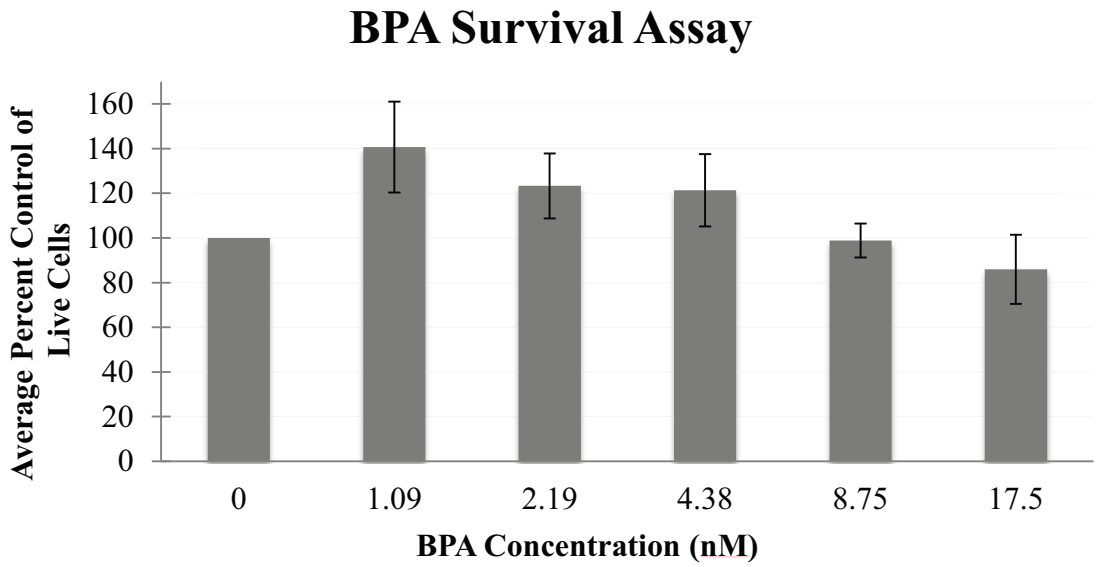

Figure 6: Effect of varying concentrations of BPA on U2OS cell growth. Numbers shown represent the mean number of live cells relative to the control in each trial (n=5) with standard error. ANOVA resulted in a p-value of 0.106, indicating no significant difference between the experimental groups.

The lowest concentrations of BPA, 1.09nM and 2.19nM, resulted in an apparent increase in U2OS cell growth to an average of 140 and 120 percent control, respectively. As the concentration of BPA increased, the number of live cells decreased. Chronic exposure to 4.38nM BPA resulted in an apparent increase to an average of 120 percent control live cells after the 24-hour growth period. The 8.75nM BPA appears to have minimal change in cellular growth, and the average percent control of live cells was 100 percent. The 17.5nM BPA exposure appears to have decreased cell growth and the average percent control of live cells at the end of the treatment was 85 percent ***(Figure 6)***.

BPA Acute Stress Response

After evaluating the effects of low levels of BPA on cell growth we then tested the short-term impact of higher concentrations of BPA on the cellular stress response. In order to carry out the acute exposure 7.0 X 10^4 U2OS GFP-G3BP cells were plated in each well of a 12-well plate and allowed to grow for 48 hours. Then two wells were

treated with each of the following concentrations of BPA for 1 hour: 500µM, 400µM, 300µM, 250µM with two wells left untreated. Cells were counted under the 40X objective (400X total magnification) and classified as either positive or negative for visible stress granules. The results of these counts, including number of total cells, number of cells with stress granules, and percent maximal stress granule formation for each trial (n=4) was tabularized and can be found in Supplemental Data ***(Tables S2)***. Percent maximal stress granule formation is a normalization of values in each trial to the maximum percent of stress granule formation obtained in that trial. The normalized values from the four trials were averaged and the mean values were graphed with standard error bars ***(Figure 7)***.

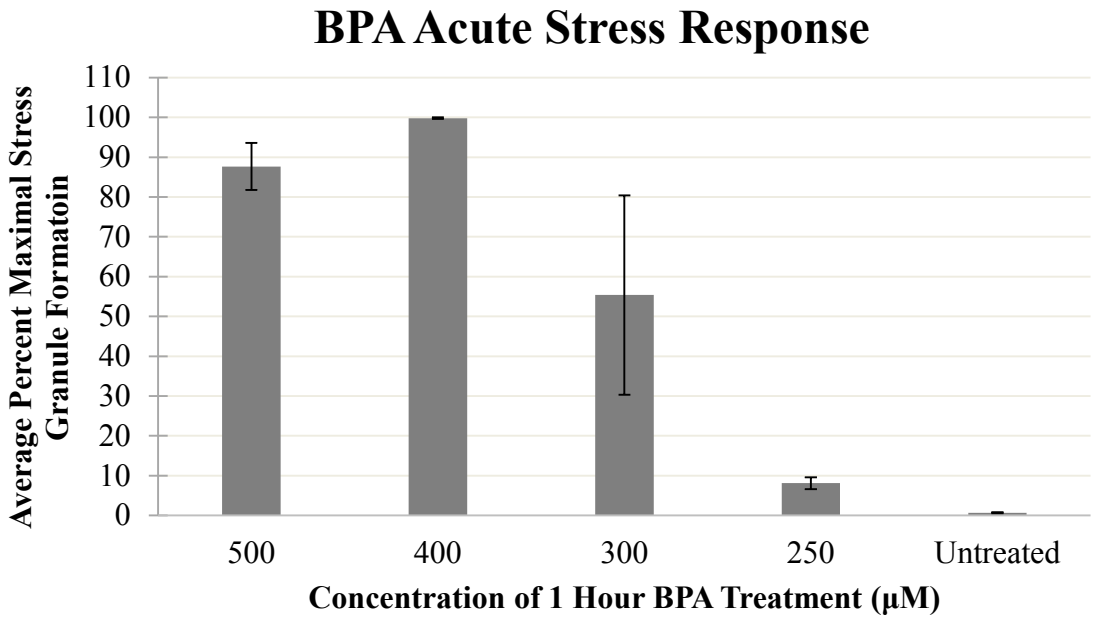

Figure 7: Effects of Various Concentrations of BPA on Stress Granule Formation During Acute Exposure. Numbers shown represent the mean percent of cells showing stress granule formation relative to the maximum number of stress granules formed during the trial from four separate trials with standard error. The p-value from the ANOVA for this data set was 2.65×10^{-5}.

The control, which received no treatment, consistently showed minimal stress granule formation. The 250µM treatment showed consistent low levels of stress granule formation. This can be observed in the graph as the data shows approximately 9%

maximal stress granule formation with a small error bar ranging from 8-10%. The 300µM treatment showed moderate stress granule formation (55% maximal stress granule formation) but with a large error bar ranging from 30-80%. The 400µM BPA exposure showed consistently robust stress granule formation as indicated by the 100% maximal stress granule formation seen in the figure. The highest concentration exposure, 500µM BPA, showed 88% maximal stress granule formation with an error bar ranging from 82-93% (***Figure 7)***. An ANOVA was performed on this data set and revealed a p-value of 2.65×10^{-5}. This p-value indicates the difference between the treatment groups is statistically significant. Two-tailed T-tests were also performed between the treatment groups to test for significance.

BPA Adaptive Stress Response

To test the adaptive stress response of U2OS-GFP-G3BP, cells were exposed to a low-level of BPA for 24 hours and then underwent a subsequent acute exposure of BPA for 1 hour. To do this, 7.0×10^4 U2OS-GFP-G3BP cells were plated on cover slips in a 12-well plate and allowed to grow uninterrupted for 24 hours. The initial BPA treatment occurred at 24 hours and remained on the cells until the conclusion of the experiment while the second BPA treatment occurred at 48 hours for one hour. Wells were treated according to the following chart (all BPA dilutions are in cultured media):

Table 1: Treatment Schedule for Adaptive Stress Response

Treatment	BPA Treatment added at 24 hours	BPA Treatment at 48 hours
No Treatment	0	0
Acute	0	400 µM
Chronic	4.38 nM	0
Chronic & Acute	4.38 nM	400 µM

Under the 40X objective (400X total magnification) cells were counted and classified as either positive or negative for visible stress granule formation. The results of these counts, including total number of cells, number of cells with stress granules, and percent maximal stress granule formation for each trial (n=5) were tabularized and can be found in Supplemental Data ***(Tables S3)***. Percent maximal stress granule formation is a normalization of values in each trial to the maximum stress granule value obtained in that trial.

The average percent maximal stress granule formation from all 5 trials was calculated for each treatment group along with standard error bar ***(Figure 8)***.

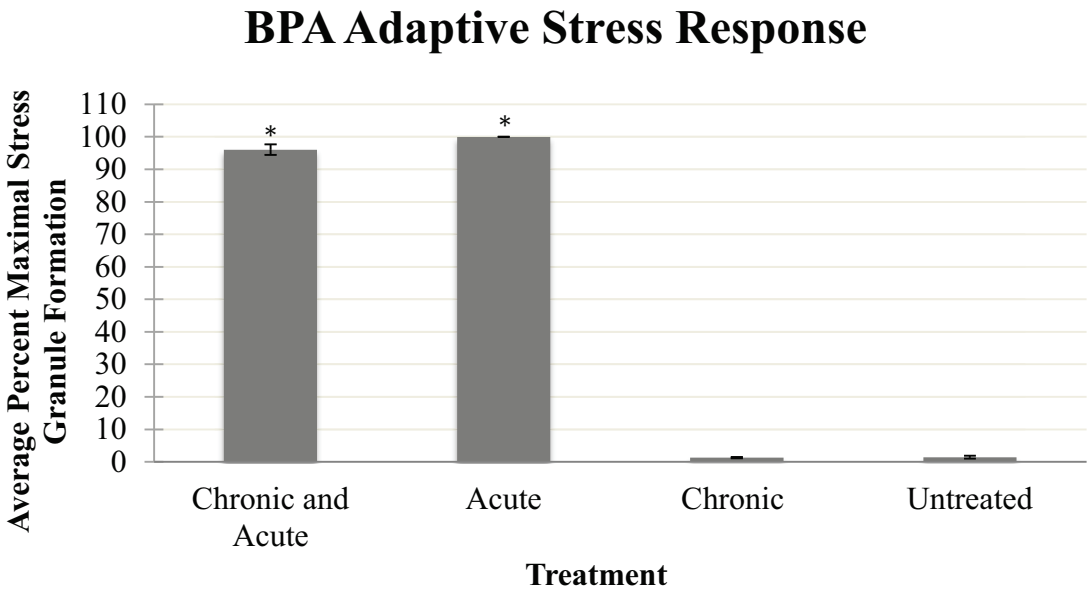

Figure 8: Effect of Chronic BPA Exposure on Stress Granule Formation During Subsequent Acute Exposure. Numbers shown represent the mean percent of cells showing stress granule formation relative to the maximum number of stress granules formed during each trial from five separate trials with standard error. Asterisk (*) indicates statistical significance according to a one tailed T test with 95% confidence (p-value of 0.037)

As can be seen in ***Figure 8***, no BPA treatment as well as low-dose chronic BPA exposure (4.38nM) had little to no stress granule formation. However, the low-dose chronic & high-dose acute BPA treatment group as well as the high-dose acute treatment group had very high stress granule formation (both above 90% maximal stress granule formation). A one tailed T test with 95% confidence resulted in a p-value of 0.037, indicating significant difference between these two experimental groups. Thus, the hypothesis was confirmed, and a chronic low-dose exposure to BPA did in fact result in less stress granule formation during a subsequent acute high-dose BPA exposure. The difference in stress granule formation was roughly 4%, and the tight error bars suggests that this result is consistent and reproducible.

Photographs of a representative field from each treatment group were then taken using the 63X oil immersion object lens at 630X total magnification, and are shown in ***Figure 9***.

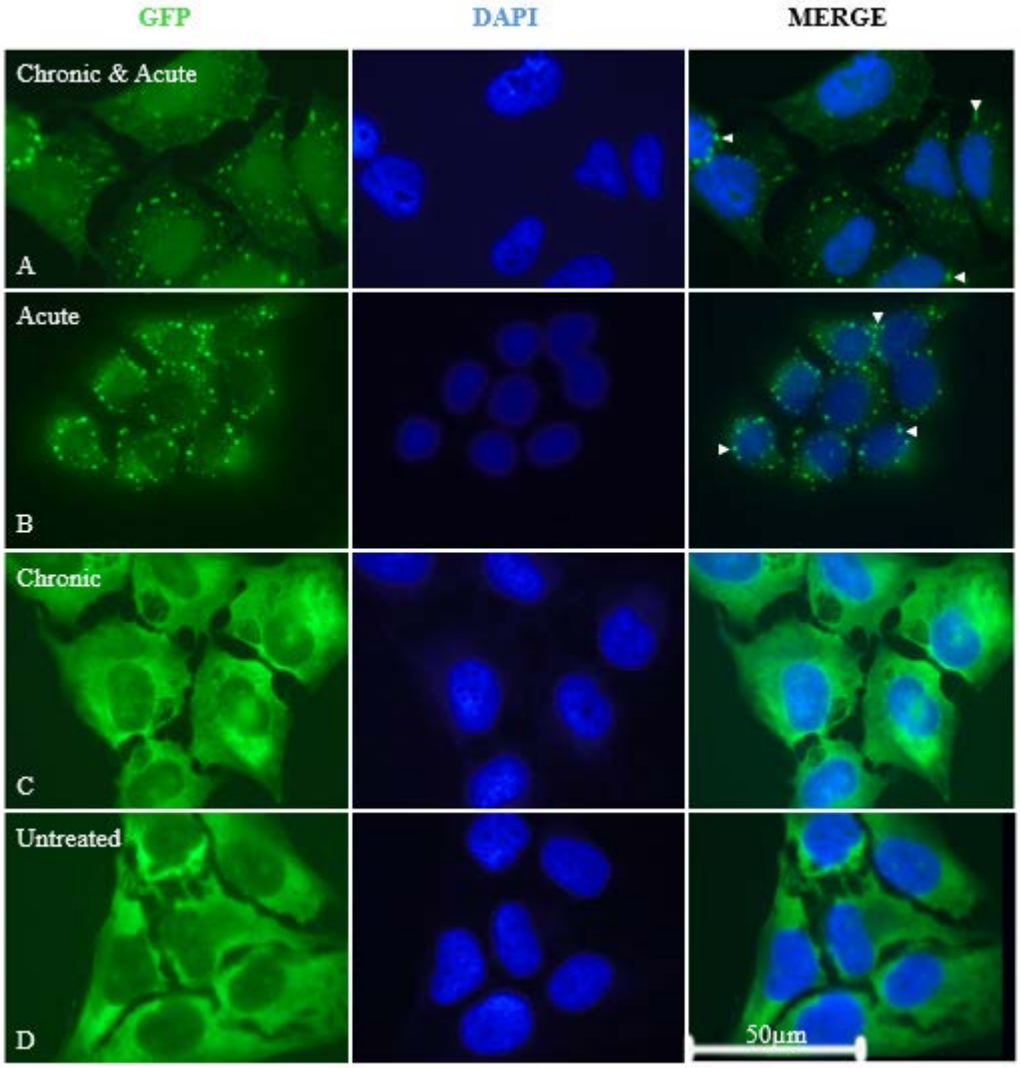

Figure 9: 630X total magnification of U20S GFP-G3BP cell(s) from the four experimental groups in the BPA Adaptive Stress Response. (A) 25 hour Chronic (4.38nM) & 1 hour Acute (400µM) BPA exposure, (B) 1 hour acute (400µM) BPA exposure (C) 25 hour exposure to chronic (4.38nM) BPA and (D) untreated. White arrows indicate stress granules.

As can be seen in the florescence microscopy images above, both the chronic & acute treatment group (A) as well as the acute treatment group (B) resulted in high stress granule formation while the 4.38 nM BPA exposure for 25 hours (C) along with the untreated cells (D) resulted in little to no stress granule formation.

DISCUSSION

Sodium Arsenite Acute Stress Response

As a positive control, a 500µM sodium arsenite 1-hour exposure was performed to ensure stress granule formation within the U2OS-GFP-G3BP cells could be visualized. Previous literature has shown that that stress granules should form in this particular cell line under these conditions (Kedersha et al., 2005b). ***Figure 5*** shows that stress granules (indicated by arrows) did in fact form and were centralized around the nucleus as expected. These results showed our U2OS-GFP-G3BP cells allowed us to visually identify stress granules using fluorescence microscopy, which would allow us to test other environmental toxins (BPA) for the formation of stress granules.

BPA Survival Assay

In order to ascertain how exposure to BPA would affect the growth of U2OS GFP-G3BP cells, a survival assay was performed. The concentrations chosen were physiologically relevant, and included the range of BPA reported to be in average healthy human serum. It was hypothesized that chronic low-dose exposure to BPA would cause a decrease in cellular growth in a dose-dependent manner. The percent control data shown in ***Figure 6*** shows that the lower concentrations, 1.09nM, 2.19nM and 4.38nM, had live cell counts that were higher than the control plate. However, as the concentration of BPA increased, the growth of the cells declined linearly from 140 percent control to 85 percent control. While these differences were not statistically significant, there was an apparent trend with proliferation at lower doses. Our cell counting method is a potential source of some variability in the data, so in order to validate this data we suggest repeating this experiment with a more accurate assay. A few examples of more accurate, non-biased, cell counting assays are MTT or crystal violet staining.

A potential spike in growth at the lower concentrations of BPA could be attributed to a biphasic dose response. This is when a low-level exposure to an environmental toxin has the opposite effect on cells as it does during a high-level exposure (Calabrese, 2013). The cell growth at lower levels of BPA exposure suggests that BPA could be interfering with cell signaling resulting in inappropriate cellular growth. This finding is especially important because it may account for some of the correlation between BPA exposure and diseases such as cancer and reproductive

disorders. The cell growth begins to decline at the higher concentrations of BPA most likely because the BPA is becoming too toxic to the cells and they are no longer able to grow.

BPA Acute Stress Response

After observing the effect of physiologically relevant BPA levels on cell growth, the next experiment was to test if an acute exposure to BPA was capable of inducing stress granules. Originally we used a BPA exposure of only 30 minutes, but that exposure time resulted in a very small number of cells with stress granules, so we increased the exposure time to 1 hour. It was hypothesized that acute high-dose exposure to BPA would result in the formation of stress granules.

One hour of BPA exposure did in fact cause stress granule formation in the U2OS GFP-G3BP cell line. This can be seen in ***Figure 7***. It is hypothesized that the formation of stress granules is caused by oxidative stress that BPA imposes on the cell because BPA has been shown to impact the levels of superoxide dismutase, catalase, glutathione reductase, and glutathione peroxidase, which are all important in the cell's oxidative stress response (Chitra et al., 2003).

The control, which received no treatment, consistently showed minimal stress granule formation. The 250µM treatment showed consistent low levels of stress granule formation. The 300µM treatment showed moderate stress granule formation (55% maximal stress granule formation). The 400µM BPA exposure showed consistently robust stress granule formation as indicated by the 100% maximal stress granule formation ***(Figure 7)***. The highest concentration exposure, 500µM BPA, also showed consistent robust stress granule formation.

An ANOVA was performed on this data set and revealed a p-value of 2.65×10^{-5}. This p-value indicates the difference between the treatment groups is statistically significant. The large error bar that accompanies the 300µM data suggests there may be a threshold for robust stress granule formation. Below this threshold some cells do begin to form stress granules but once this threshold is surpassed nearly all of the surviving cells form stress granules. This is supported by the data for the exposures above and below 300µM. The 250µM exposure would be below this threshold and results in consistent low levels of stress granule formation. The 400µM exposure is above this threshold and leads

to consistent high levels of stress granule formation. There is an apparent decrease in the percentage of cells forming stress granules during the 500µM exposure. However, the difference between 400µM and 500µM was not statistically significant as determined by a two-tailed T test with a p-value of 0.14.

A possible explanation for this apparent decrease between 400µM and 500µM could be an upper toxicity limit for stress granule formation. The upper toxicity limit of stress granule formation would prevent futile stress granule formation when cells have reached the irreversible fate of apoptosis. This data shows that acute BPA exposure does result in stress granule formation that is dose dependent.

BPA Adaptive Stress Response

After testing the impact of physiologically relevant levels of BPA on cell growth as well as stress granule formation in response to acute high-dose BPA stress, the next step was to analyze the impact of a combination of the two. Thus, cells were treated with a chronic low-level of BPA for 24 hours followed by a one hour high-level BPA treatment. 4.38 nM was selected to be the chronic low-dose concentration of BPA because this was the BPA concentration in the middle of our survival assay range (***Figure 6***). The acute BPA treatment concentration was chosen to be 400µM because it consistently formed the maximal number of stress granules in an acute 1-hour exposure (***Figure 7)***.

The hypothesis being tested was that a chronic low-dose exposure to BPA would decrease the amount of stress granule formation in a subsequent acute high-level BPA stress, known as an adaptive response. As can be seen in ***Figure 8***, the hypothesis was confirmed and the experimental group that first received a chronic low-dose BPA exposure before a subsequent acute BPA exposure had significantly less stress granule formation (roughly 4% less) than the treatment group that only received the acute high-dose exposure. Significance was supported by a one-tailed T-test with 95% confidence that resulted in a p-value of 0.037.

It is hypothesized that this decrease in stress granule formation could be attributed to the fact that the chronic low-level exposure to BPA elevates cells threshold for initiating a stress response. We believe the cells have adjusted their protein expression to acclimate to consistent low-level exposure to BPA which impacts later stress granule

formation. This adaptive response may prove detrimental because stress granules act to protect eukaryotic cells and decrease the chance of apoptosis (Arimoto et al., 2008). Thus, cells that are exposed to a chronic low level of BPA and have an adaptive response to a later acute high-level exposure to BPA would have a smaller number of stress granules and thus a greater potential for cell apoptosis.

CONCLUSIONS

During our BPA survival assay our data showed BPA levels similar to average human serum levels (1.4nM) had no significant effect on cell growth and did not result in significant stress granule formation ***(Figure 6)***. However, we observed a possible dose-dependent response to chronic exposure to physiologically relevant BPA levels on cell growth. In order to further investigate this chronic low-dose effect of BPA on cell growth, we suggest longer chronic exposure to our physiologically relevant BPA concentrations. This will allow for an amplification of the impact on cellular growth. We believe the lack of statistically significant differences between cell growths may have been due to a lack of ample time for these differences to manifest, and/or the limitations of the assay system used for the experiment.

Our acute BPA stress response assay provided data that showed high-level acute BPA exposure caused a significant increase in stress granule formation when compared to untreated cells. It also revealed a possible threshold of robust stress granule formation ***(Figure 7).*** In order to further investigate this threshold of robust stress granule formation, we recommend repeating our acute exposure with various concentrations centralized around 300µM. We suggest starting at 250µM BPA and increasing by 25µM until 350µM BPA as a possible starting point. This should allow the dose-dependent threshold to be more accurately identified.

Our final assay analyzed adaptive cellular stress response to BPA exposure. We found chronic-low level (4.38nM) BPA exposure resulted in a significant decrease in stress granule formation during a subsequent high-level (400µM) BPA exposure. In order to further investigate this phenomenon, we have a few suggested experiments. It is possible that during the adaptive stress response an increased number of cells are undergoing apoptosis because they are not forming adequate stress granules. This would decrease the number of cells negative for stress granule formation therefore increasing the overall percentage of cells positive for stress granule formation. In order to see if this is happening, a cytochrome stain could be used to identify the number of cells undergoing apoptosis during both the acute and the chronic & acute treatment. Another continuation of our experiment would be to increase the chronic exposure time to see if longer chronic low-dose BPA exposure would further decrease the stress granule

formation during subsequent high-dose acute BPA exposure. Additionally, if a more specific threshold for robust stress granule formation during acute BPA exposure is identified, this can be used in the adaptive stress response assay. By using concentrations just below the robust stress granule formation threshold the adaptive stress response can be further analyzed to see if this threshold is actually increased during an adaptive stress response. Lastly, one more future experiment could be to change the secondary acute stress to see if chronic low-dose exposure to BPA has an impact on the cellular stress response during acute exposure to other cellular stressors, such as arsenic.

SUPPLEMENTAL DATA

The following tables include the raw data from all the experiments performed. To review the methods used to collect this data, please refer to the Methodology Section of this paper.

Tables S1: BPA Survival Assay Raw Data

Table S1.1: BPA Survival Assay Trial 1

BPA Concentration (nM)	Live Cell Concentration Per Well (cells/mL)	Percent Control
17.5	1.165×10^6	88.9
8.75	1.55×10^6	118.3
4.38	2.37×10^6	180.9
2.19	2.22×10^6	169
1.09	2.66×10^6	203
0	1.31×10^6	100

Table S1.2: BPA Survival Assay Trial 2

BPA Concentration (nM)	Live Cell Concentration Per Well (cells/mL)	Percent Control
17.5	7.432×10^5	72.5
8.75	9.334×10^5	91.1
4.38	1.12×10^6	109.3
2.19	1.175×10^6	114.6
1.09	1.365×10^6	133.2
0	1.025×10^6	100

Table S1.3: BPA Survival Assay Trial 3

BPA Concentration (nM)	Live Cell Concentration Per Well (cells/mL)	Percent Control
17.5	3.84×10^5	49
8.75	6.5×10^5	82.9
4.38	7.155×10^5	91.3
2.19	8.73×10^5	111.4
1.09	9.63×10^5	122.8
0	7.84×10^5	100

Table 1.4: BPA Survival Assay Trial 4

BPA Concentration (nM)	Live Cell Concentration Per Well (cells/mL)	Percent Control
17.5	3.95×10^5	77.1
8.75	4.42×10^5	86.3
4.38	4.98×10^5	97.3
2.19	4.233×10^5	82.7
1.09	4.167×10^5	81.4
0	5.12×10^5	100

Table S1.5: BPA Survival Assay Trial 5

BPA Concentration (nM)	Live Cell Concentration Per Well (cells/mL)	Percent Control
17.5	1.11×10^6	142.3
8.75	9.03×10^5	115.8
4.38	1.0×10^6	128.2
2.19	1.08×10^6	138.5
1.09	1.27×10^6	162.8
0	7.8×10^5	100

Tables S2: BPA Acute Stress Response Raw Data

Table S2.1: BPA Acute Stress Response Trial 1

Concentration (µM)	Stress Granules Count 1	Total Count 1	Stress Granules Count 2	Total Count 2	Average % of Stress Granules	Percent Maximal Stress Granules
500	125	170	96	152	68.63354037	71.70341119
400	197	209	116	118	95.71865443	100
300	125	134	155	167	93.02325581	97.18404042
250	6	141	8	170	4.501607717	4.702957583
0	1	141	1	122	0.760456274	0.794470293

Table S2.2: BPA Acute Stress Response Trial 2

Concentration (µM)	Stress Granules Count 1	Total Count 1	Stress Granules Count 2	Total Count 2	Average % of Stress Granules	Percent Maximal Stress Granules
500	80	102	112	138	80	87.63485477
400	131	138	110	126	91.28787879	100
300	118	130	169	189	89.96865204	98.55487194
250	0	145	3	127	1.102941176	1.208201122
0	1	125	1	104	0.873362445	0.956712388

Table S2.3: BPA Acute Stress Response Trial 3

Concentration (µM)	Stress Granules Count 1	Total Count 1	Stress Granules Count 2	Total Count 2	Average % of Stress Granules	Percent Maximal Stress Granules
500	163	171	106	110	95.72953737	100
400	126	136	141	145	95.01779359	99.25650557
300	40	148	24	115	24.33460076	25.42015916
250	10	142	7	132	6.204379562	6.481154858
0	1	112	0	112	0.446428571	0.4663436

Table S2.4: BPA Acute Stress Response Trial 4

Concentration (µM)	Stress Granules Count 1	Total Count 1	Stress Granules Count 2	Total Count 2	Average % of Stress Granules	Percent Maximal Stress Granules
500	121	127	106	125	90.07936508	91.36011435
400	100	102	111	112	98.59813084	100
300	0	104	1	167	0.36900369	0.374250188
250	0	127	0	183	0	0
0	1	157	0	136	0.341296928	0.346149491

Tables S3: BPA Adaptive Stress Response Raw Data

Table S3.1: BPA Adaptive Stress Response Trial 1

Condition	Stress Granules Count 1	Total Count 1	Stress Granules Count 2	Total Count 2	Stress Granules Count 3	Total Count 3	Average % of Stress Granules	Percent Maximal Stress Granules
Chronic	2	116	1	111	0	127	0.847457627	0.860848605
Chronic and Acute	227	248	139	154	205	216	92.39482201	93.85478533
Acute	154	155	155	157	134	138	98.44444444	100
Negative Control	3	108	4	150	2	125	2.349869452	2.387000572

Table S3.2: BPA Adaptive Stress Response Trial 2

Condition	Stress Granules Count 1	Total Count 1	Stress Granules Count 2	Total Count 2	Stress Granules Count 3	Total Count 3	Average % of Stress Granules	Percent Maximal Stress Granules
Chronic	2	104	1	105	3	108	1.892744479	1.950626574
Chronic and Acute	100	106	105	118	89	104	89.63414634	92.37525173
Acute	128	129	97	105	102	103	97.03264095	100
Negative Control	0	113	0	121	2	112	0.578034682	0.595711584

Table S3.3: BPA Adaptive Stress Response Trial 3

Condition	Stress Granules Count 1	Total Count 1	Stress Granules Count 2	Total Count 2	Stress Granules Count 3	Total Count 3	Average % of Stress Granules	Percent Maximal Stress Granules
Chronic	1	106	2	100	1	128	1.19760479	1.399375163
Chronic and Acute	106	129	91	122	94	110	80.60941828	94.19035288
Acute	104	116	140	168	124	146	85.58139535	100
Negative Control	4	161	1	146	4	101	2.205882353	2.577525575

Table S3.4: BPA Adaptive Stress Response Trial 4

Condition	Stress Granules Count 1	Total Count 1	Stress Granules Count 2	Total Count 2	Stress Granules Count 3	Total Count 3	Average % of Stress Granules	Percent Maximal Stress Granules
Chronic	0	122	1	145	3	138	0.987654321	1.012040847
Chronic and Acute	126	129	160	162	119	124	97.59036145	100
Acute	100	101	124	126	112	118	97.39130435	99.79602791
Negative Control	0	108	0	114	2	133	0.563380282	0.577290906

Table S3.5: BPA Adaptive Stress Response Trial 5

Condition	Stress Granules Count 1	Total Count 1	Stress Granules Count 2	Total Count 2	Stress Granules Count 3	Total Count 3	Average % of Stress Granules	Percent Maximal Stress Granules
Chronic	2	146	3	131	0	132	1.222493888	1.26573322
Chronic and Acute	111	116	0	0	130	134	96.4	99.8096463
Acute	105	113	104	105	102	104	96.58385093	100
Negative Control	3	128	0	145	1	119	1.020408163	1.05649977

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