Effects of Chronic and Acute Arsenic Exposure On the Cellular Stress Response

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by:

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Abstract

Arsenic contamination of water supplies is a global concern. Stress granules (SGs) are cytoplasmic aggregations of RNA and protein formed in response to myriad environmental stresses, including arsenic. Acute and sub-lethal chronic levels of arsenic were tested for effects on SG formation. High level acute arsenic exposure resulted in maximal SG formation while low acute exposure had comparable results to sub-lethal chronic exposure. The results suggest that even trace amounts of heavy metals, when exposed chronically, cause considerable cellular stress.

Introduction

Normally a cell is a smooth metabolic factory. An animal cell absorbs nutrients and releases wastes. Some of the energy gained from metabolism is used to maintain the cell, prepare for division, or simply do the function it is specialized for. Certain environmental conditions can impair function and interfere with normal cellular metabolism; these are called stresses. For example, cells operate within an optimal temperature range. Above this range proteins begin to denature, so excess heat is considered a stress. Cells must adapt to the stress or take other measures to protect themselves from cellular damage, otherwise they will be unable to perform their specialized functions, or may even undergo apoptosis (Arimoto et al. 2008). One mechanism used by eukaryotic cells to protect the cell from acute transient stress is the formation of stress granules (SGs).

Stress Granules and the Cellular Stress Response

When eukaryotic cells are stressed, they form non-enclosed aggregations of protein and RNA that are called stress granules (SGs). The formation of SGs is triggered by many different stresses such as osmotic shock, heat shock, oxidative shock, and heavy metal poisoning (Anderson and Kedersha 2009). Other types of stress such as X-rays and most DNA damaging stressors do not induce SG formation (Anderson and Kedersha 2009). The RNA inside appears to be largely transcripts that are prevented from being translated immediately. The RNA and proteins in SGs display a dynamic localization, and are capable of shuttling in and out of the SG (Anderson and Kedersha 2009). Housekeeping genes are often found inside these SG, while stress related transcripts are typically excluded (Anderson and Kedersha 2009).

Due to the selectivity of SGs to exclude stress-induced transcripts, one proposed function of SGs is to sequester housekeeping mRNAs and permit the preferential translation of mRNAs necessary for cellular survival during stress (Anderson and Kedersha 2009). Also, there is considerable localization of signaling proteins involved in the stress pathways inside the SGs (Anderson and Kedersha 2009). Several studies have demonstrated that the formation of SGs protects the cell from apoptosis and allows the cell to recover from transient stress (Arimoto et al. 2008). SG formation has been associated with lower production of reaction oxygen species such as superoxide. Oxidative stress from sources such as arsenic presence can cause permanent damage; formation of SGs reduces the impact of this damage may avert apoptosis (Takahashi et al. 2013).

Arsenic

The heavy metal arsenic has many methods of toxicity. In cells, it is often found in the trivalent or pentavalent form. The trivalent form is much more toxic (Hughes 2002). Animals poisoned with arsenic often have the arsenic species arsenic trioxide, monomethylarsonic acid (MMA), arsenite, and arsenate, in descending order of toxicity (Hughes 2002). The first three are trivalent and will react with thiols. One mechanism of toxicity is the binding to thiols of the reductases such as GSH reductase. This causes the cell to lose the ability to control its redox states, and can be lethal to cells and tissues (Hughes 2002). The mechanism of arsenate toxicity is debated. One proposed action of toxicity is that it can compete with phosphate during glycolysis and create 1-arsenato-phospho-D-glycerate (Hughes 2002). 1-arsenato-phospho-D-glycerate is unstable and the arsenate hydrolyses, generating heat (Calabrese 2013). It is

unknown whether it is the generation of heat, or the metabolism of arsenite, that is the primary mechanism of arsenate toxicity (Hughes 2002).

Trivalent arsenic can also interfere with removal of 8-oxo-guanine inside the DNA double helix (Lee and Weinfeld 2004). This is a base analogue that occasionally will cause cytosine to be replaced by adenine during DNA replication, leading to base substitution mutations. While it is not thought to be a potent mutagen in and of itself, arsenic may increase susceptibility to other mutagens. For example, a class of proteins called metallothioneins (MTs) may be involved in defense against trivalent arsenic. In mice three different MT missense mutations and a promotor mutation in the gene have been traced to an increased hepatotoxic effect of arsenic (Lee and Weinfeld 2004). Cellular responses to arsenic include increased transcription of base excision repair proteins, upregulation of oxidative stress associated proteins, and upregulation of p53 (Lee and Weinfeld 2004). Upregulation of p53 appears to be linear in their entire testing range up to 500 µM arsenic (Lee and Weinfeld 2004). Cells may also spend more time in G1 in response to arsenic exposure (Lee and Weinfeld 2004). A fibroblast cell line that was deficient in radiation response, AT5BI, failed to have these responses to arsenic. However, this may be a generalized stress response separate from stress granules. P53 is also upregulated in the presence of UV and X-ray radiation, however X-ray radiation normally does not induce stress granule formation (Lee and Weinfeld 2004). While some details are known about cellar response to arsenic such as increased repairing pathway, less is known about the stress granule response to arsenic (Lee and Weinfeld 2004).

Arsenic contamination of drinking water is a major public health concern. For example, in three counties in southeast New Hampshire, it is estimated that over 49,000 people are drinking from well water containing arsenic at levels greater than the Environmental Protection

Agency's maximum contaminant level of 10 µg/L (0.133 µM) (Flanagan et al. 2014). In 2008, India's West Bengal region had 3417 villages with all available groundwater measuring over 50 µg/L (0.667µM) (Ghosh and Singh 2013). Arsenic poisoning affects many of the body systems. On the skin, there can be hyperkeratosis and gangrene of the feet (Hall 2002). In the circulatory system, exposure can cause splenomegaly and formation of regenerative (fibrotic) nodules in the liver (Hall 2002). In the nervous system there can be peripheral nerve damage and neuropathy (Hall 2002). In addition, at any exposure of arsenic, there is a weak but significant positive correlation with birth defects (Wu et al. 2011). One organ particularly affected by arsenic poisoning even at low doses is the kidney. Partial renal failure is often a symptom of arsenic poisoning, even at levels where other symptoms are uncommon (Singh et al. 2011).

Nonlinear Dose Responses

When tissues are subjected to an environmental insult, the dose dependent result is not always linear, instead it is often biphasic. Biphasic, or hormetic, dose responses occur when an outcome (survival, proliferation, production of a protein, tissue response, or animal behavior) tends to go in the opposite direction of what is expected at low doses of the molecule, compound or stimulus before reversing and going in the expected direction at higher doses (Calabrese 2013, Figure 1).
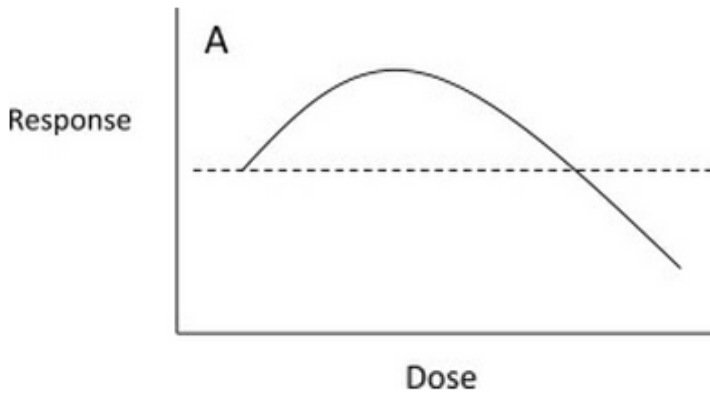
Figure 1: The most common form of the hormetic dose–response curve depicting low-dose stimulatory and high-dose inhibitory responses (Calabrese 2013).

One example of a biphasic dose response is sodium arsenite, which actually increases the survival of cultured hamster cells before a slightly larger dose causes survival rates to plummet (Calabrese 2013). The theory for biphasic response is that cells and tissues initially overcompensate for the original insult, which confers some protective benefit to the cells (Calabrese 2013). The implication for toxicology is that there is a threshold of safe levels of a cellular insult, in this case sodium arsenite, that will not result in a lower survival or function than no exposure, or, in fact, may even be beneficial or protective (Calabrese 2013). A direct relationship between SG formation and non-linear dose responses to arsenic has not yet been examined.

Examination of Chronic and Acute Arsenic Exposure and the Relationship to SG formation

Arsenic has been demonstrated to cause oxidative damage, and at higher doses decreases cell survival. The goal of this project was to determine the effects of acute and sub-lethal chronic arsenic stress on SG formation. My hypothesis is that sub-lethal chronic stress alone would induce very little stress granule formation by itself because the cells will adapt to the constant stress. However, subsequent responses to acute stress may be inhibited compared to the typical

SG response elicited by acute stress alone. I also wished to determine the threshold for decreased cellular survival in response to arsenic, and to determine whether I could observe a biphasic dose response with low dose exposure to arsenic.

Materials and Methods

Cell Culture Conditions

U2OS osteosarcoma cells with a stable integration of GFP-G3BP (Kedersha et al. 2008) were used for all experiments and were a kind gift from Dr. Nancy Kedersha, Brigham and Women's Hospital, Boston, MA. Cells were cultured in DMEM (MediaTech) with 10% FBS (Equitech), 2 mM glutamine (BioWhitaker) and penicillin streptomycin (Biowhitaker). The cells were kept in an incubator at 37^OC with 5% CO_2 as a replenishable buffer.

Cellular Survival Assay

U2OS cells were plated at a density of 4.7 x10^5 cells in 3 mL of standard culture media per well in a6-well plate. The cells were given three hours to settle, then sodium arsenite was applied to the following final arsenic concentrations (in µM): 316.0, 237.0, 178.0, 133.0, 100.0, 75.0, 51.3, 42.1, 31.6, 23.7, 17.8, and 10.0. After 73 hours, cells were trypsinized and scraped from the wells, and then counted. The procedure was performed in triplicate.

Stress Granule Assay

1.5 cm cover slips were placed inside the wells of 12-well plates. 8 x 10^4 cells were plated per well. An hour later, arsenic concentration would be brought up to the desired molarity if there was a chronic stress test. The cells were allowed 73 hours of incubation. Cells were subjected to a high level of acute stress. The arsenite concentration would be brought up to 100µM, 500 µM, or the well would get an injection of DMEM. 30 minutes after this the cells were fixed to the cover slips with paraformaldehyde and then mounted on labeled slides. The

slides were viewed and photographed under 40X magnification. The microscope was an AXIO model made by Zeiss. Each sample was counted for cells with stress granules and cells without stress granules. Each plating attempt was viewed as a unit of observation. Slides were blinded to prevent experimenter bias during counting.

Statistical Analysis

The tests performed were the student's T-test on a Texas Instruments 84 calculator. Unless otherwise stated, all results said to be statistically significant had p-value < 0.01. All comparisons stated to be insignificant had p-value > 0.05.

Results and Discussion

U2OS cells expressing a stable integration of a fluorescent SG marker protein did not exhibit a significant biphasic response to arsenic

One of the objectives of this project was to determine if there was a biphasic response to doses of arsenic. If a biphasic response did exist, there would be a dose zone where survival is not negatively affected. The level of chronic stress used for subsequent experiments would then be chosen from within that zone if it existed. To examine cellular growth under arsenic stress, U2OS (osteosarcoma) cells expressing a stable integration of a well-documented marker of stress granules tagged with GFP (GFP-G3BP, Kedersha et al. 2008) were plated in 6-well plates, exposed to increasing doses of arsenic for 72 hours, then counted to determine viability relative to untreated controls. The resulting survival curve is shown in Figure 2.
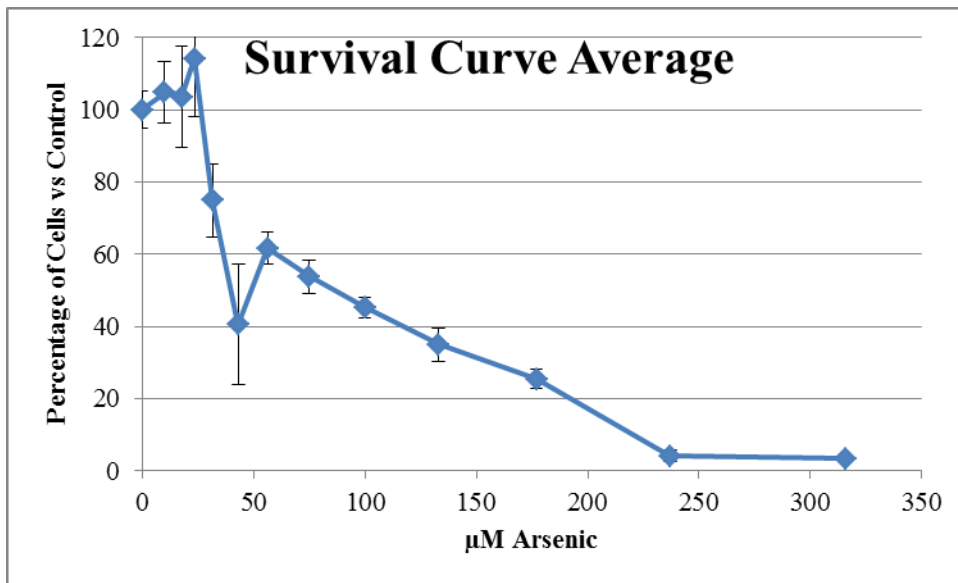

Figure 2. Survival of cells in response to arsenic concentration (as percent of control) after 72 hours of arsenic exposure. Survival was assayed by direct cell counts. Error bars are +/- SEM, n=3.

There is an increase of cell survival, and perhaps even increased proliferation, at the lower doses of arsenic, consistent with a biphasic response. However, it should be noted the standard error of the mean (SEM) bars are fairly large. While the zero dose has an SEM of 5.05% survival of the mean, the 10 µM arsenic has a SEM of 8.51%, 17.8 µM arsenic has a SEM of 14.08%, and 23.7 µM arsenic has a SEM of 16.09%. The p-values for the comparison of the control to the 10 µM arsenic, 17.8 µM arsenic, and 23.7 µM arsenic were 0.747, 0.920, and 0.150, respectively. None of the apparent increases are significant. There is not an observation of a biphasic dose response. However, the response is not linear.

The 43.2 µM arsenic group had 40.5% survival of the control ($p < 0.01$). After three days in that level of arsenic, there is indeed an observable drop in survival compared to the control at this level of stress. The 100 µM arsenic had 45.26% survival compared to the control whereas the 316 µM group had 3.33% survival compared to the control. The p-value was also below 0.01.

It should be noted that all of the nonzero doses with arsenic had at least 10 µM of arsenic. In the Indian wells previously described, the 3417 wells associated with areas with arsenic poisoning had at least 0.667 µM. This means that in this survival curve, cells have full viability in lab dishes at higher doses than arsenic poisoning is observed in the field. Or another way to put it, osteosarcoma cells survive at levels of arsenic that has deleterious effects on whole humans. One organ particularly affected by arsenic poisoning is the kidney (Singh et al. 2011). Since the U2OS line is an osteosarcoma, perhaps the reason for this difference is the different cell type. Another possibility is that it takes more than 72 hours of exposure for low levels of arsenic to cause cell death.

The hypothesis of a biphasic response was not supported by the data. One possible reason is that 72 hours was simply insufficient exposure time. Another possibility is that there was a

true biphasic response, but due to insufficient sample size the averages of each individual dose sample are not representative of population response.

In the survival assay, there was at first an inability to successfully trypsinize the cells for counting. The procedure was modified to scrape the cells from the wells after adding the trypsin. While this did yield cells to count, there was a difference between the apparent confluence of the wells under the microscope and the cell count. A possibility for future assays is the use of MTT assays to quantify cells numbers; the MTT assay uses a eukaryote's respiratory chain to quantify viable cells (Freimoser et al. 1999).

Chronic sub-lethal arsenic exposure elicits a SG response akin to a moderate acute dose exposure

In order to understand the response of cells to low doses of arsenic, a SG formation assay was performed. As shown in Figure 3, some wells were incubated in 3 µM arsenic for 72 hours, and others were not. The acute arsenic dose, if used, was added 30 minutes before fixing the cells.
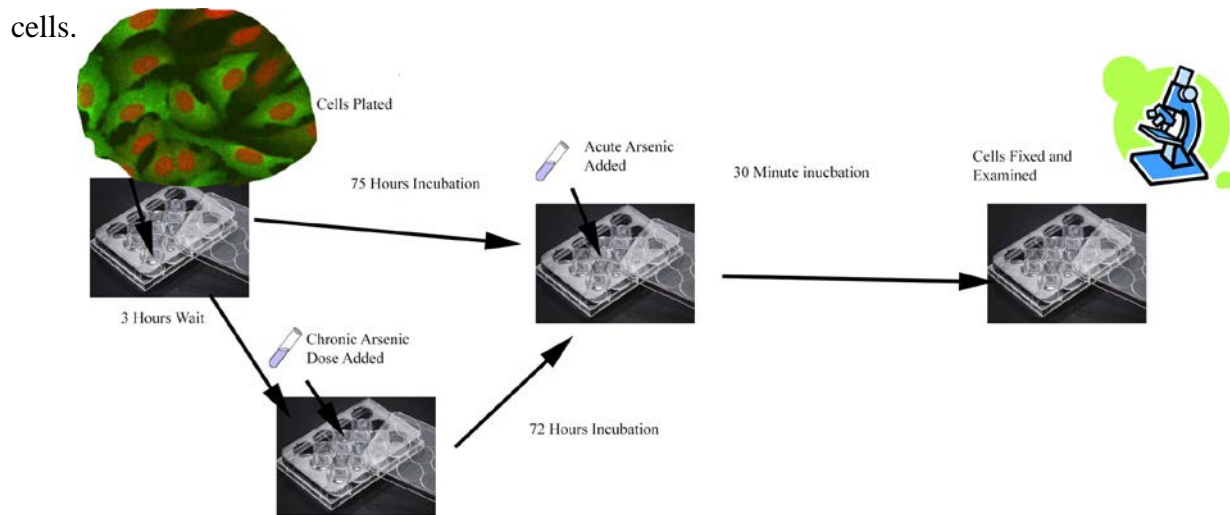

Figure 3. Flowchart for Stress Granule Assay. An appropriate number of cells were plated on each well. If the cells were to receive a chronic dose of arsenic, arsenic was added to the medium three hours after plating. There was incubation for 72 hours, for a total of 75 hours. Cells without this treatment were simply incubated over 75 hours. Acute arsenic treatment involved adding arsenic to the medium half an hour before fixing cells to the coverslips.
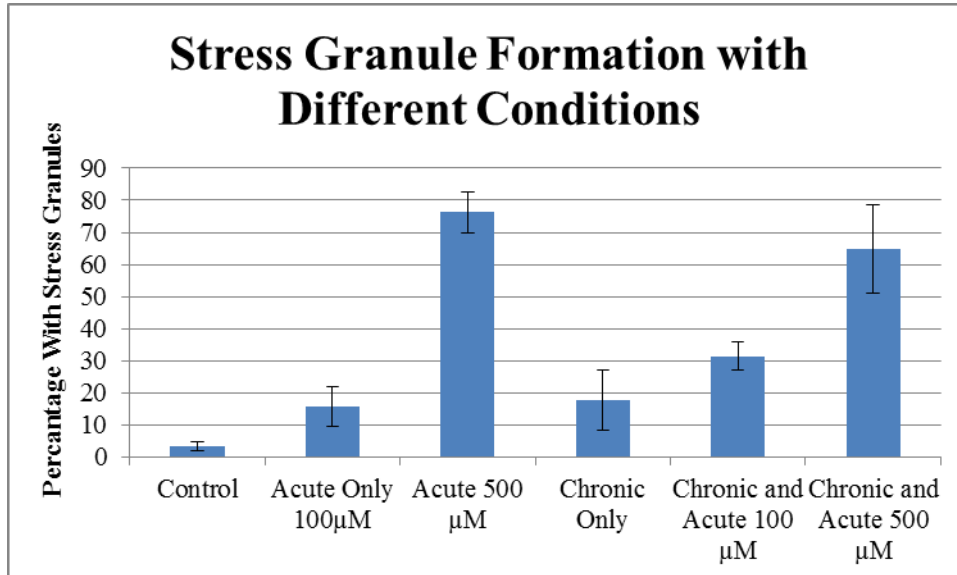
Figure 4. Stress granule formation in response to chronic and acute arsenic exposure. Cells were observed and scored for presence of stress granules (as percentage of total cells scored). All treatment conditions are statistically different from control (P<0.01). Error bars are +/- SEM, n=3.

The results of the stress granule analysis are shown in Figure 4, and representative fluorescence microscopy images of each sample are shown in Figure 5. A first look at the graph in Figure 4 suggested that chronic stress increased the baseline of SG response. Then when 100 µM of arsenic was added, the proportion of cells with stress granules increased by the same amount in addition to the existing SGs already present. At 500 µM, there is a saturation of response and chronic stress before the acute stress did not significantly change the proportion of cells with SGs. This is reinforced by Figure 5F, both acute stress and chronic stress had SG formation and with 500 µM acute arsenic exposure, most cells have them.
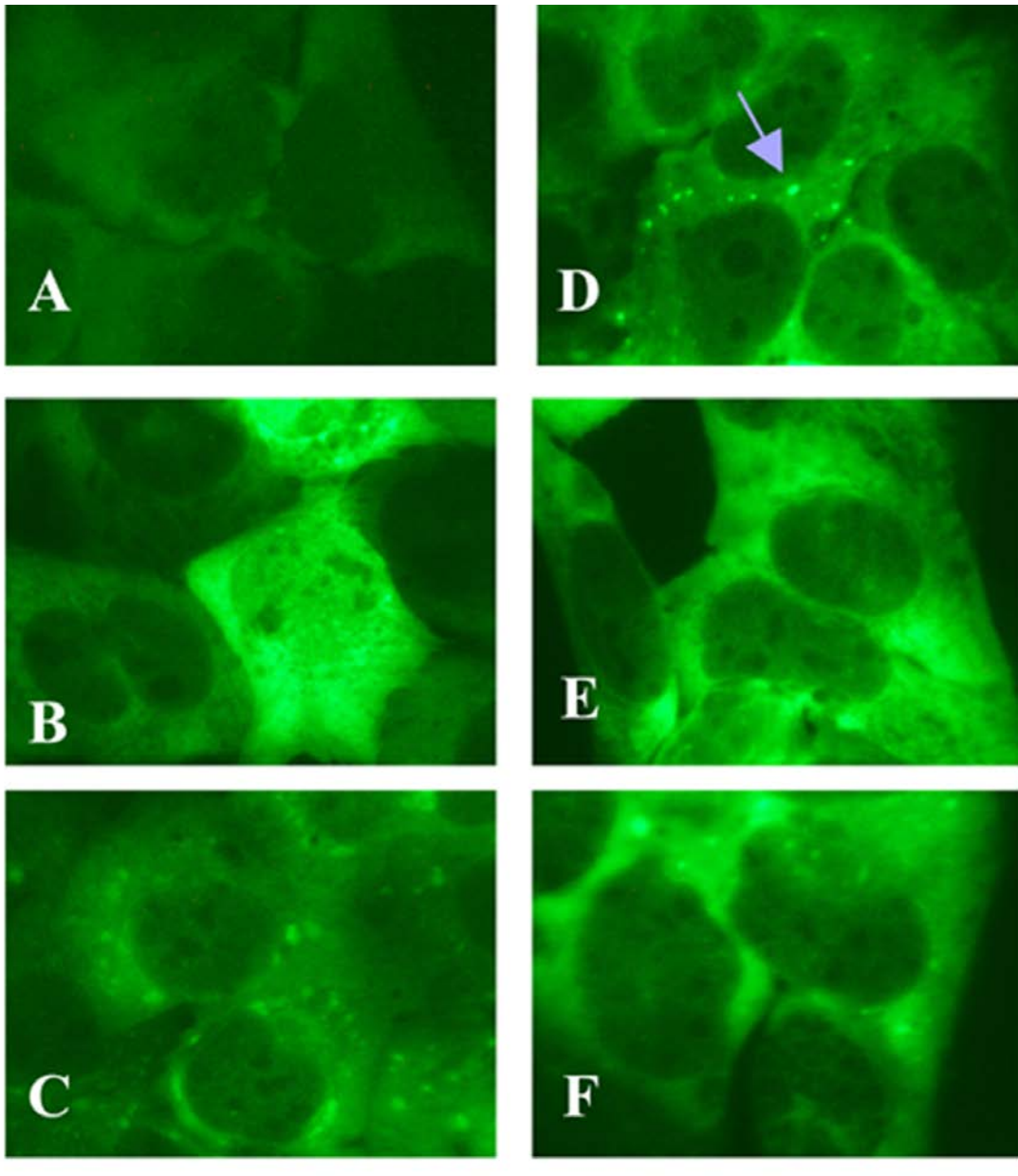
Figure 5. Fluorescence microscopy of stress granules under different arsenic conditions. In panel D an arrow points to a stress granule. The panels are as follows: A) control, B) acute 100 µM arsenic, C) acute 500 µM arsenic, D) chronic arsenic only, E) chronic and acute100 µM arsenic, F) chronic and acute100 µM arsenic.

All of the groups in comparison with the "Acute 500 µM" had p-value < 0.01, except for the chronic stress followed by 500 µM acute arsenic. The tests support the graph appearance. This is seen in the slides samples themselves. Figure 5A, the control, had little SG formation. Figures 5B, 5D, and 5E have a minority cells with SG formation. The high arsenic dose, seen in Figures 5C and 5F, show most of the cells having SGs. There is no significant difference in stress granule formation between chronic stress alone and the addition of 100 µM acute stress afterwards, despite the fact that the jump was similar in size to the acute 100 µM over the control. To get significantly more SGs, an additional 400 µM was needed. What this is suggesting is that either 100 µM acute stress or chronic low level (3µM) stress induces some level of SG formation that is only partially saturated, and further SG formation can be elicited with higher acute doses.

As previously stated chronic arsenic stress alone and 100 µM acute arsenic stress did not have a statistically different proportion of cells with SGs. Despite the fact that the chronic dose was only 3 µM for 75 hours, previously established to be sub lethal to this cell line under similar conditions, the cells were stressed to a similar amount as cells that did receive a moderate level of arsenic 100 µM, over half an hour. This suggests that at low and moderate doses of arsenic the cells' response is cumulative.

At the highest concentration of acute arsenic exposure of 500 µM, there is an apparent decrease of SG formation if chronic stress was previously applied as seen in Figure 3. The SEM bar for chronic and acute 500 µM stress is wide. One reason the SEM bars were fairly large in Figure 4 is that the sample size is very small. In particular, the chronic and acute 500 µM stress had one sample with a similar percentage of cells showing stress granules as the maximum sample of the 500 acute µM stress displayed. With the outlier removed, the differences between

the two conditions would be larger, possibly significant, however there was nothing wrong with the sample that warranted ignoring it. There are three possible explanations. One is that there is a difference and more replicates would detect the difference. Another is that there simply is not any difference in the conditions for SG formation. A third possibility is apoptosis occurring as the cells because highly stressed in the face of high arsenic exposure after being pretreated. As currently observed, the two conditions are not statistically significant, in fact the p-value= 0.759. Perhaps the chronic stress is inducing apoptosis in a sub-population of cells upon high dose acute exposure. It may also be the case that chronic exposure desensitized another sub-population of cells such that the percent of cells with SG upon subsequent acute high dose exposure in the remaining cell population is decreased.

Conclusions

No biphasic response was observed between arsenic dose and survival. From 0 to 23.7 µM of arsenic for 72 hours, there were no significant differences in survival. At higher doses, not only was there significantly lower survival than the control, there was a significant difference between the highest dose and the intermediate dose.

Chronic stress increases the SG response to a similar degree as acute 100 µM arsenic exposure. The similar response is thought to mean that at the lower doses the stress of arsenic exposure is cumulative. At high concentrations of arsenic exposure, previous chronic exposure did not impair the SG formation and stress response.

The fact that cell survival was observed over existing arsenic guidelines does not mean existing EPA thresholds are conservative. As previously mentioned, it has been observed various organs were affected above current safe thresholds, especially the kidney. The osteosarcoma line's survival is not indicative of the behavior of other cell types. Also, longer chronic exposures, as may be experienced by persons exposed to arsenic in the water supply, may have different outcomes in the osteosarcoma cells.

The cells subjected to the chronic dose of arsenic formed SGs despite the levels being sub lethal. The cells were in a moderate state of stress. Perhaps over time, the state of chronic stress might induce apoptosis over time. Alternatively, the cells might be able to cope with a low level of stress for prolonged periods of time and the existing SGs offer sufficient protection form receiving enough damage to induce apoptosis. If this is the case, then the cells could be pushed beyond that limit with additional stress. In the SG assay, the cells pretreated with chronic arsenic exposure might have had a higher rate of apoptosis once exposed 500 µM acute arsenic. The

cells stressed the most would have been marginally surviving and not able to handle additional stress. This could explain the high SEM bar for that particular condition.

For future experiments, the direct continuation would be to get more replicates for the SG assay, to narrow the SEM bars, particularly in the chronic and 500 µM acute arsenic exposure. Once this is done and any differences (if existing) are detected, the next topic is examining the relationship between the SG formation observed and apoptosis. The chronic and 500 µM acute arsenic condition in particular is interesting and may have above baseline level of apoptosis. A third future experiment can be the examination of arsenic and nephrocytes, since the kidneys are sensitive to arsenic poisoning. Do the SGs provide some degree of protection from low chronic exposure to arsenic?

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