The Effect of Acute Heavy Metal (Cu And Cd) Toxicity on Ros Generation, Apoptosis, and Intracellular Glutathione Levels in Saccharomyces Cerevisiae

THE EFFECT OF ACUTE HEAVY METAL (Cu AND Cd) TOXICITY ON ROS GENERATION, APOPTOSIS, AND INTRACELLULAR GLUTATHIONE LEVELS IN *SACCHAROMYCES CEREVISIAE*

by

JESSE GARDNER

Under the Direction of John E. Houghton, PhD

ABSTRACT

Heavy metal contamination is of growing concern as industrialization causes increasing levels to be released into the environment affecting all organisms. Cellular damage and coping mechanisms due to heavy metal toxicity vary depending upon the species of metal and organisms exposed. It is assumed that cellular damage can be attributed to the destructive effects of metal-induced ROS production, which is ameliorated, by the non-protein tripeptide glutathione, cycling between reduced (GSH) and oxidized (GSSG). We investigate the effects of acute cadmium and copper treatment on *Saccharomyces cerevisiae and* determine some of the immediate cellular responses to the exposure of these metals and demonstrated ameliorative effects of free form amino acid precursors (FAAPP). FFAAPs reduced cellular oxidative stress and served to offset the immediate oxidative stress that the presence of both metals caused within the cell within the first hour of exposure. FFAPs effected cellular responses differentially for each metal.

INDEX WORDS: Glutathione, *Saccharomyces cerevisiae,* acute heavy metal, free form amino acid precursors, ROS, apoptosis.

THE EFFECT OF ACUTE HEAVY METAL (Cu AND Cd) TOXICITY ON ROS GENERATION, APOPTOSIS, AND INTRACELLULAR GLUTATHIONE LEVELS IN *SACCHAROMYCES CEREVISIAE*

by

JESSE GARDNER

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

Master of Science

in the College of Arts and Sciences

Georgia State University

2019

THE EFFECT OF ACUTE HEAVY METAL (Cu AND Cd) TOXICITY ON ROS GENERATION, APOPTOSIS, AND INTRACELLULAR GLUTATHIONE LEVELS IN *SACCHAROMYCES CEREVISIAE*

by

JESSE GARDNER

Committee Chair: John Houghton

Committee: Julia Hilliard

P. C. Tai

Electronic Version Approved:

Office of Graduate Studies

College of Arts and Sciences

Georgia State University

December 2019

DEDICATION

This paper is dedicated to my mother Melody Garrison and my father, Jeff Gardner, without whom none of this would be possible. Furthermore, I want to dedicate this to my nephew Aiyden; he is my inspiration and reason for everything that I do.

ACKNOWLEDGEMENTS

I want to acknowledge Dr. John Houghton for his infinite patience and outstanding guidance. I can always count on Dr. Houghton to challenge my thinking and expand my understanding beyond what I thought possible. Dr. Therese Pool opened the world of molecular biology to me in the best possible way. Dr. Hillard is always quick with a smile and encouraging words, and she has made my cloudy days a little brighter. Dr. Tai methodical way of thinking helped illuminate the trees when I was lost in the forest. My lab mates provided glad support and ensured the process was as painless as possible and helped in too many other ways to enumerate here.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS I

LIST OF FIGURES IV

LIST OF ABBREVIATIONS VI

1 INTRODUCTION 1

Purpose of the Study 10

2 MATERIALS AND METHODS 11

2.1 Strains 11

2.2 Buffers and Solutions 12

2.3 Growth Rate and Determination of appropriate FFAAP concentration 13

2.4 Determination of Intracellular ROS 14

2.5 Analysis of caspase-dependent and independent cell death 14

2.6 Cell lysis and Glutathione Analysis 15

3 RESULTS 17

3.1 Determination of optimal FFAAP concentrations for the growth of *S. cerevisiae* at millimolar concentrations 17

3.2 Analysis of intracellular ROS generation after acute cadmium or copper treatment 19

3.3 Acute heavy metal treatment causes apoptosis in *S. cerevisiae* 22

3.4 The effect of cadmium and copper exposure on glutathione biosynthesis after one-hour treatment in *S. cerevisiae* 27

3.5 Investigation of FFAAPs supplementation on ROS generation in *S. cerevisiae*... 28

3.6 Effect of FFAAPs on apoptotic levels in *S. cerevisiae* 32

3.7 Glutathione biosynthesis for *S. cerevisiae* cultured in FFAAPs media 36

4 DISCUSSION 38

Redox-inactive versus redox-active heavy metals 38

ROS accumulation due to heavy metal treatment 39

Apoptotic response due to acute heavy metal treatment 40

Glutathione response after acute heavy metal treatment.. 41

The role of FFAAPs in elevating glutathione 43

Glutathione levels as a result of FFAAPs supplementation 44

The effects of FFAAPs on ROS generation after acute heavy metal treatment............... 45

The effects of FFAAPs supplementation on apoptosis 46

Future directions 47

5 CONCLUSION 48

REFERENCES.. 49

APPENDICES 56

Appendix A 56

Appendix B 59

LIST OF FIGURES

Figure 1. Apoptosis triggers in yeast. 3

Figure 2. Haber-Weiss reaction catalyzed by Cu^{2+}.. 5

Figure 3. Potential sources of ROS generation relevant to apoptosis in yeast, and cellular mechanisms for mitigation of ROS effects. 7

Figure 4. Natural sources of glutathione pool reduction.... 8

Figure 5. Glutathione biosynthesis pathway. 10

Figure 6. Growth curves depicting the influence of FFAAPs treatment on the growth rate of S. cerevisiae.. 18

Figure 7. A temporal determination of the immediate generation of ROS from yeast cells following acute treatment with cadmium. 20

Figure 8. ***A temporal determination of the immediate, generation of ROS from yeast cells following acute treatment with cadmium.*** 22

Figure 9. Evaluation of activated yeast caspase following acute exposure (1-hr) to the heavy metals, cadmium and copper. 24

Figure 10. Analysis of correlation between FAM-FLICA stained cells and PI stained cells following 1-hour treatment with heavy metals cadmium and copper in S. cerevisiae. 26

Figure 11. Assessment of intracellular glutathione levels in yeast following acute exposure (1 hr.) to cadmium or copper.... 28

Figure 12. Temporal determination of immediate generation of ROS from yeast cells cultured in FFAAP following acute (1-hr) treatment with cadmium. 30

Figure 13. Temporal determination of immediate generation of ROS from yeast cells cultured in FFAAP following acute (1-hr) treatment with copper............................ 31

Figure 14. Supplemental FFAAPs treatment induces a slight reduction in the population size of cells undergoing apoptosis following acute heavy metal treatment for 1h. . 33

Figure 15. Analysis of PI as a marker for apoptosis in yeast supplemented with FFAAPs following 1-hour treatment with heavy metals cadmium and copper in S. cerevisiae. 35

Figure 16. FFAAP supplementation changes glutathione biosynthesis in S. cerevisiae following cadmium or copper treatments of 1h. 37

LIST OF ABBREVIATIONS

FFAAPs (Free-Form Amino Acid Precursors), 10
FLICA (Fluorochrome-Labeled Inhibitors of Caspase Activity), 14
GSH (Glutathione, Reduced), 8
GSSG (Glutathione disulfide, Oxidized), 8
PB (Phosphate buffer), 12
PCD (Programmed Cell Death), 2
PPP (Pentos-phosphate pathway), 47
ROS (Reactive Oxygen Species), 3

1 INTRODUCTION

Simple eukaryotic organisms have been shown to be reliable models for studying the underlying cellular mechanisms used by higher-order eukaryotic cells to increase survivability in a stressful environment. The yeast *Saccharomyces cerevisiae* has successfully been used to understand cellular processes such as gene regulatory elements (Johnston, 1987), MAP Kinase signal transduction (Whitmarsh & Davis, 1998), heat shock response(s) (Verghese, 2012), and disease progression using cryptic orthologs between simple models to complex eukaryotes (McGary, Park, Cha, & Marcotte, 2010). These represent but a small sampling of examples whereby information that was gleaned by studying *S. cerevisiae* has been extrapolated into higher-order eukaryotes. Numerous studies have demonstrated the effectiveness of *S. cerevisiae* as a model largely due to previous genomic (Goffeau *et al*., 1996) and proteomic work on *S. cerevisiae*, the ability of yeast to reproduce sexually or asexually, and the ability of researchers to force haploid states within the model (Gershon, 2000). Yeast has been shown to respond to environmental stressors with gene products which have been conserved among higher-level eukaryotes.

The yeast, *S. cerevisiae*, provides a suitable candidate for laboratory investigations owing to the short generation time of about 90 minutes, small size, and ability to grown in multiple media types and carbon sources. Large quantities of cells can be cultured from a 10 μL inoculation of starter culture in YEPD after 16 - 18 hours, allowing for quick investigation of numerous experiments without the laborious prospect of maintaining small numbers of slowly reproducing models. Furthermore, the small nature and sheer numbers of *S. cerevisiae* as a model system allow for ease of disposal and very little down-time in the event of a colony collapse; one can streak plates from frozen stocks and have cells to analyze in four days.

Apoptosis is a programmed cell death (PCD) characterized by specific morphological changes including chromatin condensation and fragmentation, cellular volume decrease, and finally blebbing of the cell into numerous fragments that can be absorbed by other cells, or simply remain in medium (Farrugia & Balzan, 2012; Galan *et al*., 2001; Mazzoni & Falcone, 2008). This blebbing process is important because it sequesters potentially dangerous chemicals from the media where a growing colony may exist. Apoptosis is important for higher-level eukaryotes for such programs as morphological development, brain development, and defense from cancer (E. Y. Chen, Fujinaga, M., & Giaccia, A. J., 1999; Kuan, 1999; Lowe & Lin, 2000). This program is irreversible once begun with the activation of the sole yeast caspase protein, Yca1, via a number extrinsic and intrinsic factors such as toxic compounds, elevated cytosolic heavy metal concentrations, DNA damage, or ROS accumulation due to age, mutation, or normal metabolic processes (Mazzoni & Falcone, 2008). Figure 1 (Carmona-Gutierrez *et al*., 2010) below displays common routes by which apoptosis is induced in *S. cerevisiae.*

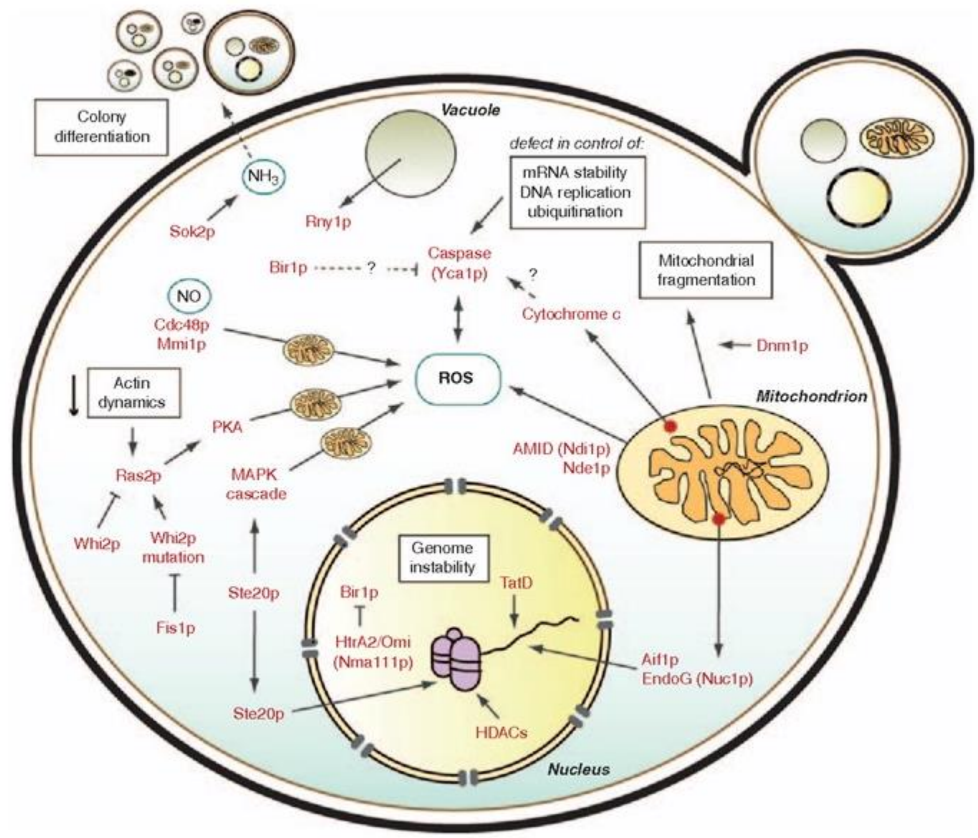

Figure 1. Apoptosis triggers in yeast. *Proteins and pathways involved in yeast apoptosis are displayed. (Camarona-Guiterrez et al., 2010)*

Stressors exist ubiquitously throughout the environment, including but not limited to xenomorphic and naturally occurring toxic compounds, ionic dysregulation, radiation, and normal metabolic function (Evans, 1984). Stressors such as heavy metals exert their effects upon the cell by increasing highly reactive superoxide ($^{o}O_2^{-}$), hydrogen peroxide (H_2O_2), and hyperoxide (^{o}OH). These oxides are collectively known as Reactive Oxygen Species (ROS), and exert damaging effects on cellular components including protein carbonylation (Cabiscol Català, 2000), lipid peroxidation (Marisa Repetto, 2011), and increased mutation rates (Emerit, 1994). Multicellular organisms tend to accumulate heavy metals in organ-systems such as the heart, kidney, liver, and skeleton (Singh, Gautam, Mishra, & Gupta, 2011) collecting damaging cations in crucial systems. The effects of assault by heavy metal cations such as lead, cadmium, and copper on organisms are of increased importance as environmental pollution by heavy metals accumulate worldwide due to mining, industry, and electronic manufacturing and disposal (Su, 2014). Therefore, organisms must exhibit numerous techniques for survival in an ever-changing and assaulting environment.

Heavy metal pollution negatively impacts all organisms in a food web, from the microbiome to top consumers (Su, 2014). Furthermore, heavy metals accumulation can occur through direct absorption at all trophic levels; studies in aquatic systems have shown that heavy metals such as mercury and cadmium enter at the microplankton level and accumulate through predation up the food chain (Chen, Stemberger, Klaue, Blum, Pickhardt, & Folt, 2000). Many experiments in heavy metal toxicity involve chronic exposure, occurring over a protracted period. The dearth of information about acute heavy metal intoxication at subtoxic concentrations leads to the question of how yeast respond under heavy metal assault within the first hour of exposure.

Heavy metals are categorized into two subgroups with regards to the ability of the said metal to generate ROS. Metals such as lead and cadmium are referred to as Redox-inactive metals. Generally, cells treated with these cations will increase ROS within the cytosol; however, it is thought the mechanism of ROS generation occurs when these metals replace canonical metals in the active site of enzymes releasing redox-active generating metals into the cytosol. Cadmium is an indirect-ROS generating heavy metal and was investigated during this series of experiments. Cadmium does not directly increase ROS within the cells after 30- or 60-minute of metal assault, yet the cells still undergo apoptosis beyond levels seen in the untreated groups. It is probable that replacement of redox-active metals in enzymes with cadmium is responsible for apoptosis in chronic exposure, but this mechanism is unlikely the cause of apoptosis under acute conditions, where there is less time involved in the treatment, as fewer redox-active cofactors are forced into the cytosol under acute treatment.

The other subgroup of heavy metals is referred to as redox-active metals and include metals such as copper, chromium (VI), and iron. These metals can increase ROS concentrations in solution via Fenton-like reactions. Copper catalyzes the production of ROS in the Haber-Weiss reaction as an example, copper (II) is reduced to copper (I) in the presence of an electron donor; copper (I) will then oxidized in the presence of H_2O_2, producing hydroxyl radical and a hydroxide ion (Fig. 2). The hydroxyl radical can participate in further redux reactions within the cell and copper (II) is available for further ROS production.

$$^{o}O_2^{-} + H_2O_2 \xrightarrow{Cu^{2+}} {}^{o}OH + OH^{-} + O_2$$

Figure 2. Fenton-like reaction catalyzed by Cu^{2+}; *where an electron donor such as superoxide or an oxygen singlet produces a hydroxyl radical and a hydroxide ion.*

Organismal studies of heavy metal maladies tend to focus on chronic exposure, which leads to the question of what mechanisms for handling heavy metal assault within the first hour of introduction are possessed by yeast? Work by colleagues within this lab showed that there are transcriptional changes and oxidative damage to proteins within the first 15 minutes of assault. Furthermore, transcriptional upregulation favors chaperone proteins, and oxidative damage is such that glycolytic proteins are somewhat more damaged (Nargund, 2014). Furthermore, there is an increase of at least 2-fold in cystine synthesizing enzymes following acute cadmium at 1 mM levels (Vido *et al.*, 2001). Therefore, the focus of these experiments was to question the mechanisms *S. cerevisiae* employ to increase survivability within the first hour of treatment.

Oxidative stress due to increasing intracellular ROS generation has been shown to lead directly to caspase-dependent apoptosis; high levels ROS have been shown to activate caspase via a signaling cascade (Mazzoni & Falcone, 2008) cementing the cell to apoptosis. Therefore, it was surprising that a one-hour exposure to the indirect-ROS generating metal cadmium-induced apoptosis above background when it was initially discovered in my lab (Nargund, Avery, & Houghton, 2008). There was some variation in apoptotic populations, depending on the age of the cells, but apoptotic populations between about 8% and 10% arise when cells were rested in YEPD medium.

As heavy metals accumulate within organisms, deleterious effects begin to appear at different concentrations depending upon cation species and organism assaulted. Early work on heavy metal-induced intracellular damaged has indicated that cadmium causes mutations in chromosomal and mitochondrial DNA, lipid peroxidation, protein carbonylation, and a secondary increase in ROS as cadmium replaces canonical metal cofactors within active sites of enzymes (El-Demerdash, Yousef, Kedwany, & Baghdadi, 2004). Copper is known to lead to direct-ROS evolution as described by the Haber-Weiss reaction causing protein, lipid, and DNA damage (Milne, Nicotera, Orrenius, & Burkitt, 1993).

Direct-ROS generating heavy metals exert biological stress by causing protein oxidation, lipid peroxidation, and mutations that can be mitigated with the enzymatic functions of superoxide dismutase (SOD) and methionine sulfoxide reductase (MSR) which are used to reduce cytosolic ROS levels (Sumner *et al*., 2005); these enzymes help to prevent random oxidation of proteins by removing cytosolic reducing agents. Furthermore, the Sumner group noted that the proteins targeted for carbonylation under chromium assault are random, but heat shock proteins are moderately favored as targets for carbonylation. It is understood that in biology form determines function and that the tertiary structure of proteins is determined by the chemical nature of the side chain; addition of nonpolar methyl groups will invariably change the chemical nature of bound amino acids which leads to changes in tertiary structure, binding potential, and/or catalytic function depending on the location of methyl inclusion. Cellular mechanisms of ROS generation (Fig. 3) (Bragado *et al.* 2007) is displayed below.

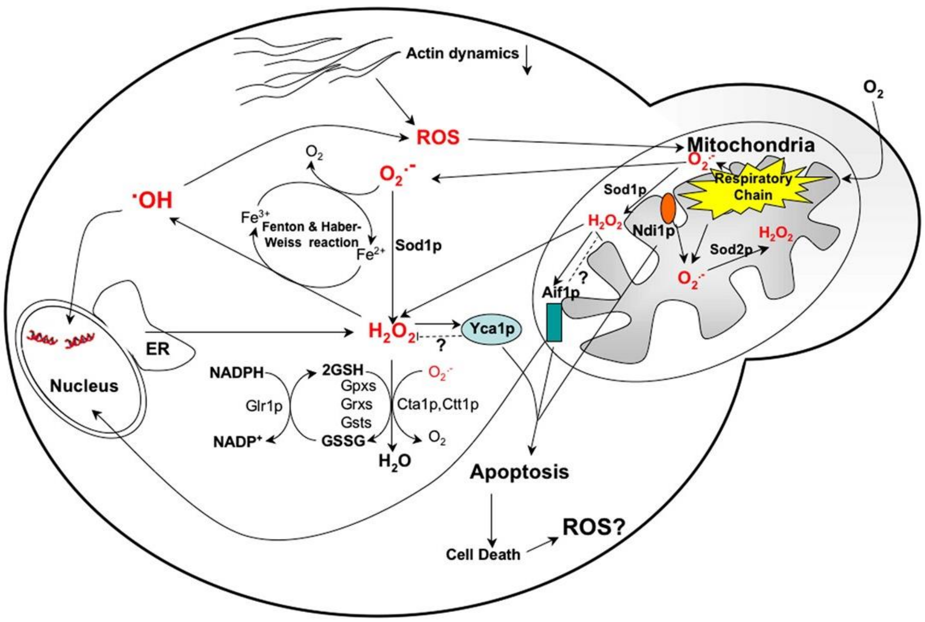

Figure 3. Potential sources of ROS generation relevant to apoptosis in yeast, and cellular mechanisms for mitigation of ROS effects. *Natural sources of ROS are highlighted in red. (Bragado, et al. 2007)*

One mechanism for managing heavy-metal assault is for a cell to change the expression profile of proteins. The cell can increase expression of enzymes to decrease ROS levels such as superoxide dismutase, γ-ECS, glutathione reductase as well as increased expression of proteins which may be oxidatively damaged, providing the cell possesses mechanisms sensitive enough to determine the location and extent of the damage. It is therefore important to examine expression profiles for cells under heavy metal assault. Historically, qPCR has been used to examine changes in expression patterns; however, this method relies on enzymatic processing which increases the time of assay and introduces variations that make analysis between runs difficult. Furthermore, the same data can be prepared and run by different groups with wildly different results. A newer method for expression analysis is Nanostring, which uses a series of six fluorescent “bar-codes” attached to oligonucleotides. The labels hybridize to target RNA and are visually inspected by the equipment, and counts are provided directly.

Glutathione (GSH) is a tripeptide consisting of the amino acids glutamine, glycine, and cysteine and is used with a series of enzymes to convert cytosolic ROS into more benign oxidative species. Free thiol groups on two GSH molecules are covalently attached using ROS, such as a hydroxyl radical as an electron donor with the aid of glutathione peroxidase, producing one molecule of glutathione disulfide (GSSG). One molecule of GSSG can then be recycled into two GSH molecules using glutathione reductase and NADPH as an electron donor, as shown in the equation below (Fig. 4a). Certain redox-inactive metal ions such as Pb^{2+}, Cd^{2+}, and Hg^{2+} show high affinity for thiol binding and are capable of depleting glutathione pools by conjugation with the thiol groups on GSH (Fig. 4b) (Rubino, 2015).

A

$$2GSH + H_2O_2 \xrightarrow{\text{GSH Peroxidase}} GSSG + H_2O + \frac{1}{2}O_2$$

$$GSSG + NADPH \xrightarrow{\text{GSH Reductase}} 2GSH + NADP^+$$

B

$$RSH + M^{n+} \leftrightarrow RS\text{-}M^{(n-1)+} + H^+ \qquad (1)$$

$$RS\text{-}M^{(n-1)+} + RSH \leftrightarrow (RS)_2\text{-}M^{(n-2)+} + H^+ \qquad (2)$$

$$n\ RSH + M^{n+} \leftrightarrow (RS)_n\text{-}M + n\ H^+ \qquad (3)\ \text{global}$$

Figure 4. Natural sources of glutathione pool reduction.

Two reduced glutathione (GSH) is oxidized by GSH peroxidase *using hydrogen peroxide as an electron donor, producing one molecule of oxidized glutathione (GSSG), water and an oxygen singlet. Oxidized glutathione is reduced by GHS reductase using NADPH as an electron donor, producing two molecules of GSH and $NADP^+$(A). (B) Metal ions (M) such as cadmium can deplete thiol-containing biochemicals by conjugation (Rubino, 2015).*

In addition to glutathione's ability to reduce free radical species as a singular electron donor, GSH will also directly conjugate with cadmium along with other metals (Rubino, 2015), producing bis(glutathionato)-cadmium, which can then be "pumped" into vacuoles for sequestration via YCF1-mediated transport in *S. cerevisiae* (Li *et al*., 1997). The mechanism by which this cellular reaction occurs in response to cadmium is unclear; currently, no enzymes have been identified in the production of bis(glutathionato)-cadmium. *S. cerevisiae* has no such mechanism for sequestering copper involving glutathione. Instead, yeast tend to increase the expression of copper transport proteins that increase the efflux of copper to the outside of the cell (Lu, 1999). It is to be expected, therefore, that the two metals might promote differing responses upon acute exposure may involve discrete use of available glutathione (GSH) pools. Furthermore, it has been proposed that one can use the ratio of GSH/GSSG as a determinant of oxidative stress in higher eukaryotes (Asensi *et al.* 1999; Zitka *et al.* 2012).

Glutathione biosynthesis proceeds through a dual-enzyme pathway, whereby γ-glutaamylcystine synthetase (γ-ECS) first combines glutamine and cysteine on the γ-carbon of glutamine to form γ-glutaamylcystine (γ-EC); glutathione synthetase, the second enzyme in the pathway, combines γ-EC with glycine producing GSH (Noctor, 2002) (Fig. 5), and GSH can conjugate with other cysteine residues and/or cadmium cations to protect the cell from oxidative stress.

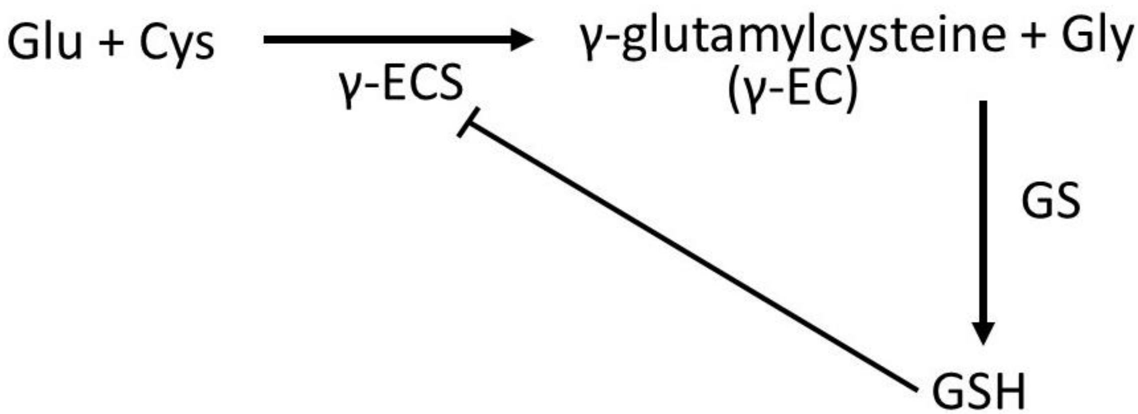

Figure 5. Glutathione biosynthesis pathway. *Glutathione is biosynthesis proceeds through a bi-enzymatic pathway. Cysteine is condensed to the γ-carbon of glutamic acid by γ-glutamyl cysteine synthetase, first. Next glutathione synthetase condenses γ-EC and glycine producing GSH, which will competitively inhibit γ-ECS in a feedback inhibition loop. (Noctor, 2002)*

The question arises: Can the levels of GSH be forcibly upregulated within the cell by providing the substrates for glutathione synthesis and/or might an increase in glutathione precursors within the cell enhance intracellular biosynthesis of glutathione (the utility of GSH) to mitigate the cellular damages that result from heightened ROS? To investigate these questions free-form amino acid precursors (FFAAPs) for GSH were used as a supplement in the culture media. In this study, FFAAPs were provided in the form of ProImmune® (*The ProImmune Company, NY, USA*) which is a dietary supplement, specifically formulated to provide enhanced basal levels of glycine, L-cystine, and glutamine (in a 1:0.5:1 mole ratio) to the diet (Crum, 2007; Lawrence & Burk, 1976). Selenium is a cofactor of glutathione reductase and is provided via selenomethionine as a supplement in trace amounts.

Purpose of the Study

This study is designed to address the hypothesis that the acute effects of cadmium and copper in yeast are different and that the two metals induce heterogeneous responses within the cell; i.e. that both metals induce apoptosis but potentially through disparate mechanisms. Additionally, the study is designed to test the effects of FFAAPs treatment on the respective, acute cellular response(s) to acute heavy-metal exposure by manipulation of the intracellular glutathione levels via increased intracellular biosynthesis of glutathione, considered to be one of the central cellular responses to ROS resulting from the presence of high levels of heavy metals, such as cadmium and copper within the cell. Ultimately this study is undertaken to gain a better understanding of some of the mechanisms simple, single-celled eukaryotic organisms, such as *S. cerevisiae*, apply to offset acute exposure to heavy metals, and possibly provide new treatments for heavy-metal-induced disorders in humans.

Apoptosis has previously been shown to occur in subpopulations of *S. cerevisiae* after only one-hour treatment with either 8 mM copper (II) or 50-80 μM cadmium (Nargund, 2014) and was believed to be a result of ROS generation (Bragado, Armesilla, Silva, & Porras, 2007; Thayyullathil, *et al*., 2008; Oh, & Lim, 2006). This study also attempts, therefore, to determine the extent to which intracellular levels of ROS predicate the acute cellular responses to heavy metals, such as cadmium and copper, after only one hour of cellular exposure to these metals. Finally, this study seeks to test the effects that increasing the levels of glutathione precursors (in the form of FFAAPs supplementation) would have on the ability of these cells to offset the effects of these heavy metals in *S. cerevisiae*.

2 MATERIALS AND METHODS

2.1 Strains

Saccharomyces cerevisiae BY4741 (MATα, his3D1, leu2D0, met15D0, ura3D0) was obtained from Euroscarf (Frankfurt, Germany). Experimental cultures were inoculated from 24 h starter cultures derived from multiple colonies and grown overnight to mid-exponential growth

(OD600 ~ 2.0) at 30 ^{o}C with shaking (120 rpm) in YEPD broth, supplemented with $KH_2PO4:K_2HPO4$ phosphate buffer (PB) at a final concentration of 100 mM. When necessary cadmium (II) nitrate [$Cd(NO3)_2$], copper nitrate [$Cu(NO_3)_2$], and/or FFAAPs [Gly, Glutamate, Cystine 1:1:0.5 and trace selenium] were added in increasing concentrations to growing cultures (final concentration of 80 µM, 8 mM, and 2 mM, respectively).

2.2 Buffers and Solutions

All buffers and solutions were made as described below and, whenever possible, used as stock solutions. Stable solutions were stored as stocks at 4 ^{o}C when not in use. Cadmium (II) nitrate (Alpha Aesar, Cat # 41732-18) was produced as100 mM stock solution, allowing for the addition of microliter volumes to growth media, with a final concentration in media of 80µM. Copper nitrate (Sigma Aldrich, 229636-25G) was produced as a 1 M stock solution, with a final concentration in media of 8 mM. Potassium buffer was produced as a 1 M stock solution; 0.5-mole potassium phosphate monobasic (Fisher Bioreagents, Cat # AC424200250**)**, and 0.5-mole potassium phosphate dibasic (VWR, Cat # 71003-450). Buffered YEPD media was prepared in a final concentration of 100 mM KH_2PO_4/K_2HPO_4. Lysis buffer (5% Metaphosphoric Acid (MPA) (*Sigma-Aldrich, USA*), 3% glycerol, 1x Tris, pH = 8.8) was produced fresh the day of lysis and was cooled to 4 ^{o}C before use. Free Form Amino Acid Precursor (FFAAP) solutions were produced immediately prior to use in growth media.

Moreover, special consideration was undertaken to ensure that Free Form Amino Acid Precursor (FFAAP) supplement would remain in aqueous solution (see Results Section 3.1). To this end, 5 ml of diH_2O was used to dilute 5.5 mL of NaOH 12N, to which 1g of FFAAPs (ProImmune™, solid phase) was added, and the mixture vortexed vigorously for about 20 seconds. To this suspension, 3.5 mL of HCl 12 N was carefully added, mixed through inversion, and allowed to cool to room temperature. The final concentration and volumes of this FFAAPs stock solution, were 200 mM and 15 mL, respectively; pH ~ 8.5. The FFAAP solution was added to the growth medium at least 20 min before inoculation with yeast, which were subsequently allowed to grow to an OD_{600} 2.0 - 2.5. Initially, all medium treated with FFAAPs was buffered by incubating YEPD in 5% CO_2 at 30^{o}C for 12 hours before treatment (see Results Section 3.1). This method of buffering media, however, proved to be unreliable as FFAAPs would precipitate out of solution during cell cultivation. Ultimately, therefore, the YEPD growth medium, which was used for all yeast growth was buffered with a 50/50 molar ratio phosphate buffer of 100 mM KH_2PO_4/K_2HPO_4, pH = 6.8, as described previously. Also, it was noted that both FFAAPs and PO_4 were found to influence the uptake of heavy metals by *S. cerevisiae,* as results were variable between experimental procedures until it was decided to wash cultured cells prior to metal treatment. Thus, it was deemed necessary that all cells, which were harvested from buffered YEPD growth medium, were immediately washed twice in unbuffered YEPD, before being resuspended in unbuffered YEPD prior to treatment with heavy metal.

2.3 FFAAP Dose-Response Analysis for Growth Rate

To establish the optimum dose of FFAAP, a dose-response analysis was performed using growth rate as an output measure. The rate of cell growth was determined by monitoring density of cells over time, using an Eppendorf Biophotometer plus. To analyze growth rates of cells yeast cultures were grown for ~15.5 hours to an OD_{600} ~1.0 to in phosphate-buffered YEPD medium (see preceding section of Materials and Methods, and Results Section 3.1). After this time, the population density of growing cells was periodically assessed at OD_{600} by taking samples every 30 min for 6 h. in the presence of FFAAP. All doubling times were calculated by identifying two timepoints within the cell populations' exponential phase and applying the following equation

$$\frac{(T2 - T1) * \log{(2)}}{(\log(OD2) - \log(OD1))}$$

2.4 Determination of Intracellular ROS

Cells were assayed for the presence of ROS by growing cells, as described above, with or without the addition of that FFAAPs concentration that was established as nontoxic (LD_{50}). Cadmium (II) nitrate [$Cd(NO_3)_2$] or copper nitrate [$Cu(NO]_3)_2$] was added from stock solutions to appropriate concentrations previously stated. From 100 mL of overnight culture (OD_{600} ~1.7), 2.5 mL samples were aliquoted and treated with 5 µL of CellRox Deep Red fluorescent probe for 30 min. Following staining, cells were washed (5000 x g, 4^{o}C suspended in PBS two times) and resuspended in appropriate 5 mL of 0.8% saline. A 1 mL aliquot of untreated cells was collected for t=0. Subsequently, 1 mL aliquots were collected at 30, and 60 minutes post treatment. Cells were then washed and assayed using the BD FACSAria II flow cytometer with excitation/filter at 633, and 660/20 nm. (*BD Biosciences, USA*)

2.5 Analysis of caspase-dependent and independent cell death

Flow Cytometry was accomplished using BD LSRFortessa (*BD Biosciences, USA*) for 10,000 total events. Cells were treated with Fluorochrome-Labeled Inhibitors of Caspase Activity (FLICA) conjugated with one of two fluorochromes, SR-FLICA or, FAM-FLICA activated caspase-specific dyes as previously defined (Nargund, 2014), where the cells were incubated in the FLICA dye during a three-hour-refractory period in YEPD, before being diluted 1:10 and analyzed on the flow cytometer. Cells were excited at 561 nm of light, and emission was assessed with a 610/20 nm filter for SR-FLICA. Other cells were treated with FAM-FLICA and/or propidium iodide (PI) to a final concentration of 5 mg x μL^{-1} for 3 h. and 45 min in YEPD respectively before analysis on the flow cytometer; excitation and filters used for FAM-FLICA and PI are 488 nm and a filter of 530/30 nm, and 561 nm and a filter of 610/20 nm respectively. Fluorescent microscopic analysis of cells and FAM-FLICA fluorescence was confirmed using an Axio Observer Z1 (*Carl Zeiss Microscopy, LLC, USA*) with a 40x oil-emersion lens at various time points.

2.6 Cell lysis and Glutathione Analysis

Following metal treatment (when appropriate), the cells were washed three times as previously described; the final resuspension for GSH assay was done with 3 mL of fresh lysis buffer. Samples (1.5 mL of cells resuspended in lysis buffer) for GSH assay were lysed in a 2.0 mL conical beater tubes with 0.5 mm silica beads, using a mechanical cell breaker on the "homogenize" setting. The actual lysis was carried out in 5 x 30 seconds bursts, with each burst followed by a 30-second rest period on ice. The lysate was centrifuged at 14,000 xG for 5 minutes, and the supernatant collected for assay. Prior to assaying for glutathione levels, a 10 mL aliquot of each set of treated cells was put aside for cytometric evaluation to confirm that appropriate levels of apoptosis had occurred. These cells were washed as described above with the final resuspension being completed in 10 mL fresh YEPD + PB, and cells were incubated, as previously described for three hours undergoing cytometric analysis.

Furthermore, an additional 20 µL of each sample was taken and put aside for quantitation using a Nexcelom Cellometer Auto 2000 (*Nexcelom Biosciences, USA*) and the small cell propidium iodide preinstalled protocol. Total glutathione levels were assayed using Enzo Life Sciences Glutathione (GSH/GSSG), detection kit per manufacturer's protocol, except where described. Five milliliters of resuspended cells were collected, spun down as described above and resuspended in 1.5 mL of lysis buffer. This solution was serially diluted to match the sensitivity of the assay, and the dilution values of 1:50 and 1:100 were most reliable concerning the whole-cell lysate. Oxidized glutathione was determined via the addition of 4-vinylpyridine to block free thiols on GSH within the solution for one hour before the assay, GSSG values can be subtracted from total glutathione levels to calculate GSH/GSSG ratios.

Total glutathione (Gtot) concentrations were calculated on a per well basis following manufacture's protocol, in Excel. In order to compensate for the variable growth rates of *S. cerevisiae* under disparate treatments, it was decided to calculate the glutathione levels on a per-cell basis. Calculations proceeded first by converting the derived concentration (pM) to molar (M); the molar concentration per well was converted to moles of glutathione per well by multiplying by 2.0×10^{-4} L (total volume of well). Moles-per-well values were then converted to molecules of GSH per well by multiplying by Avogadro's number (An). Molecules of GSH per cell were calculated by dividing this number by cell count (CC) (total cells divided by dilution factor), and moles of GSH per cell were subsequently calculated by dividing by Avogadro's number. To calculate the molar concentration of GSH per cell, moles of GSH x $cell^{-1}$ were divided by the average volume of *S. cerevisiae* (4.2×10^{-14} L). Finally, the molar concentration was converted to nano-molar. The total equation is displayed below. Mathematically the steps involving Avogadro's number can be removed, but due to a quirk of Excel it was required to add these steps.

$$Glutatione\ per\ cell\ (M) = \frac{(Gtot * 0.0002 * An)}{(CC * An * (4.2 * 10^{-14}))}$$

3 RESULTS

3.1 Determination of optimal FFAAP concentrations for the growth of *S. cerevisiae* at millimolar concentrations

Free-form amino acid precursors, as provided by ProImmune®, proved to be intractable to solubilization in the growth medium, due in part to the low solubility of cystine in water. Initial attempts to solubilize ProImmune® were provided by Mugda Vasireddi, a post-doctoral researcher in Dr. Hilliard's laboratory, who worked to find an appropriate pH for a FFAAPs solution suitable for culturing mammalian cells. However, this 200 mM stock solution had a pH approximating 8. It was discovered that the media required acidic buffering to remain in solution within media containing actively growing yeast, during additional work at various concentrations of this stock. Metabolic by-products of S. cerevisiae growth naturally increases the pH of unbuffered media, and this strain grows most efficiently at a slightly acidic pH. The addition of FFAAPs to unbuffered media changed the pH of the media to a moderately basic pH ~ 7.5, this coupled with metabolites further increased the pH of media to about 8, retarding the growth of yeast and precipitating FFAAPs out of solution.

Initially, buffering attempts with carbon dioxide proved to be somewhat effective; YEPD flasks were incubated in an atmosphere with 5% CO_2 for 12 hours before FFAAPs treatment and introduction of cells. Even so, this method for keeping ProImmune® in solution proved to be unreliable, making growth conditions and data acquisition difficult to replicate. Ultimately, dissolving the YEPD media in a 50/50 molar ratio phosphate buffer of 100 mM KH_2PO_4/K_2HPO_4 proved to be more effective, as this ratio provided a reproducible pH of 6.8, which was far more appropriate for normal yeast growth. To determine the maximal concentration of FFAAPs at which *S. cerevisiae* could be successfully cultured, a series of growth curves were generated, using FFAAP concentrations ranging from 0 - 4 mM. The doubling time for cells under all conditions was calculated to approximate 120 min except for 3 mM FFAAP which was about 110 minutes (Fig. 6). Even so, at 3 mM FFAAPs concentrations, and more so at 4 mM, there was a 'fuzzy' precipitate appeared at the bottom of the flask after around 18 h. of growth. Cells failed to grow in the presence of 5 mM FFAAP; furthermore, at this concentration, a heavy precipitate appeared 21 hours post-inoculation.

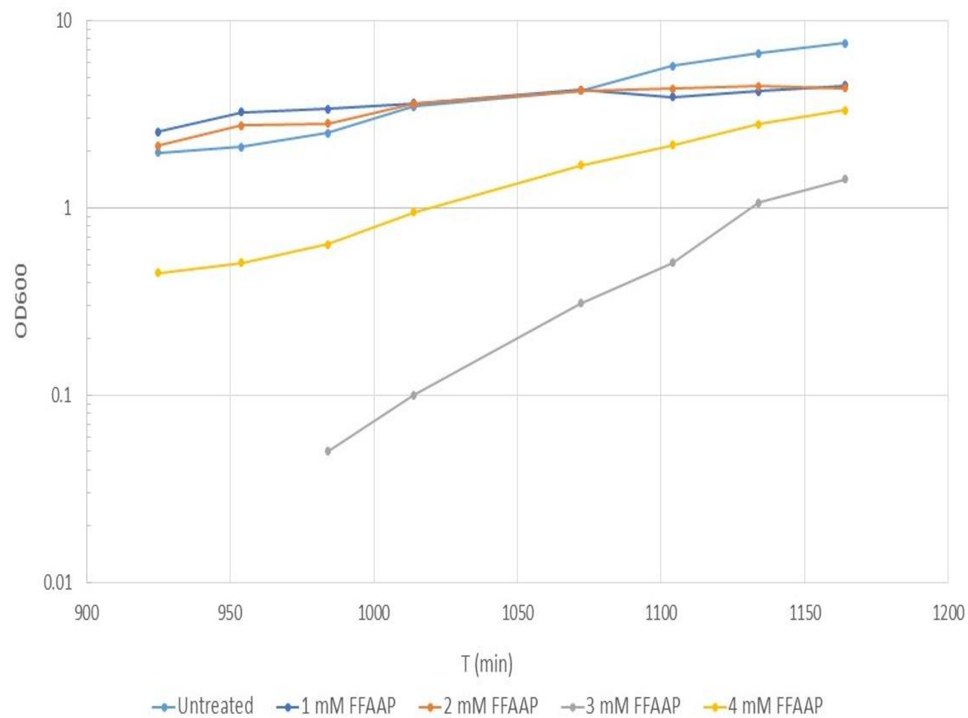

Figure 6. Growth curves depicting the influence of FFAAPs treatment on the growth rate of S. cerevisiae. *Yeast grew for 21 hours in phosphate-buffered YEPD with 0-4 mM FFAAP. Eppendorf Biophotometer plus provided OD_{600}. 2 mM FFAAP is the highest concentration that is subtoxic. FFAAP precipitated from solution at 4 mM concentration around hour 18. Precipitation of FFAAPs began at 3 mM concentrations; thus 2 mM FFAAP was chosen as maximal supplementation.*

The optimal concentration of FFAAPs in phosphate-buffered media that retained FFAAPs in solution, and which did not affect growth rates of the yeast, was determined to be 2 mM (Fig. 6). Again, while cell growth rate was demonstrably affected at 4mM FFAAPs, the solubility of FFAAPs at 3mM proved to be variable. Thus, in all subsequent analyses on *S. cerevisiae* grown in media that utilized FFAAPs, 2 mM FFAAPs was used in a YEPD medium buffered with 100 mM phosphate buffer (pH = 6.8).

3.2 Analysis of intracellular ROS generation after acute cadmium or copper treatment

Intracellular ROS generation was analyzed using CellROX deep red fluorescent probes. Following the addition of redox-inactive (cadmium) and redox-active (copper) heavy metals, the exposed cells were assessed for the presence of ROS after 0, 30, and 60 min. (Fig. 7). Background ROS levels were shown to be about 0.4% (Fig. 7A). Generation of ROS in cells exposed to cadmium remained at near background levels even after 30 min and 60 min of treatment, with little to no change in the size and complexity of the population of treated cells with those of control population (Fig. 7A-B).

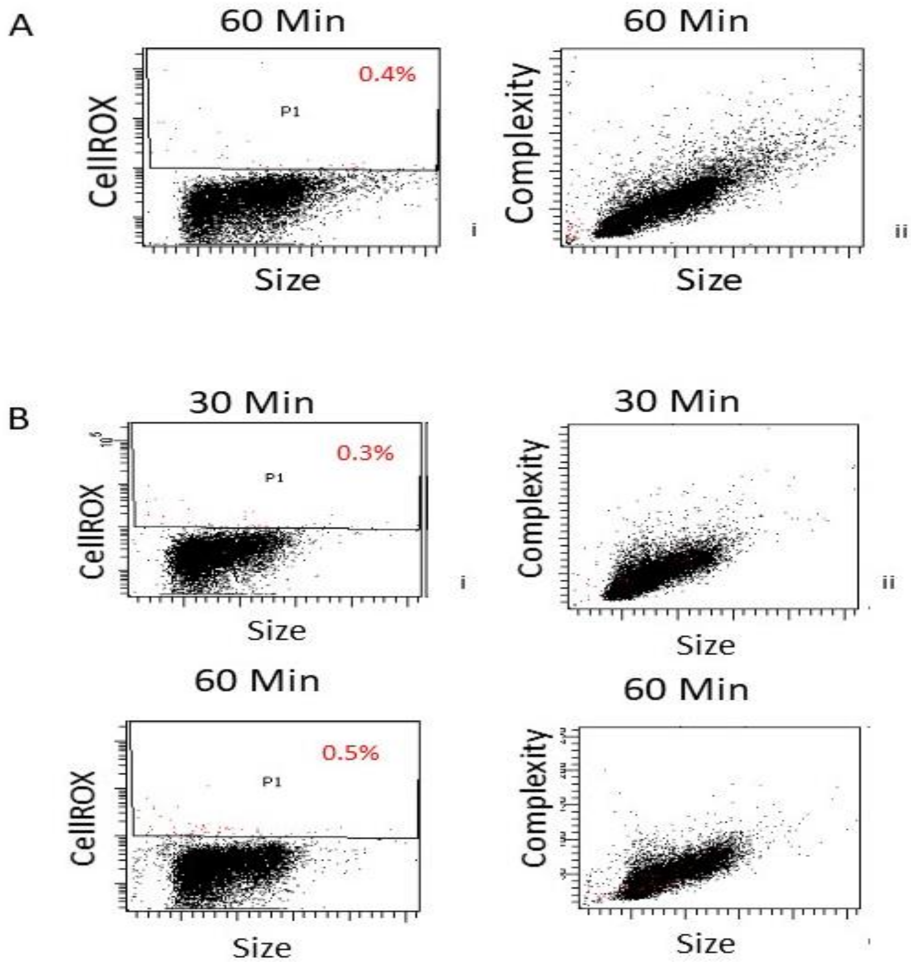

Figure 7. A temporal determination of the immediate generation of ROS from yeast cells following acute treatment with cadmium. *Representative data of flow cytometric analysis of CellRox deep red dye oxidation in cells treated with (A) 50 µM PO4 as a control, (B) 80 µM cadmium nitrate for 30 min and (C) 80µ M cadmium nitrate for 60 min. For each set of cells, cytometric evaluations included a comparison of CellRox dye oxidation vs cell size(forward scatter) -denoted bye numerals 'i', and an analysis of the same population of cells comparing complexity (side scatter) vs forward scatter (cell size) denoted by numerals 'ii'. In all cases, ROS levels were denoted by % values of the total population.*

In a similar manner, ROS generation of cells exposed to 8mM copper for 30 and 60 min. were analyzed (Fig. 8). For cells treated with 8 mM copper (II), the subpopulation of yeast that showed high ROS levels was about 30.0% after 30 min. of treatment, and about 10% after 60 min. of treatment. The subpopulation of CellROX positive cells were moved toward the lower left quadrant in the size vs complexity graph following 30-minute treatment relative to the untreated group, indicating that these cells were smaller and less complex than the rest of the population. Following 60 minutes of treatment, the population of cells with high ROS were located toward the bottom left portion of the graph (Fig 8B). The size and complexity of the population changed from the untreated group at both time points, with dye bound cells becoming smaller and less complex when compared to those of the untreated population; the ‘halo’ of larger cells decreased after 30 minutes of treatment and decreased further after 60 minutes of treatment (Fig. 8B).

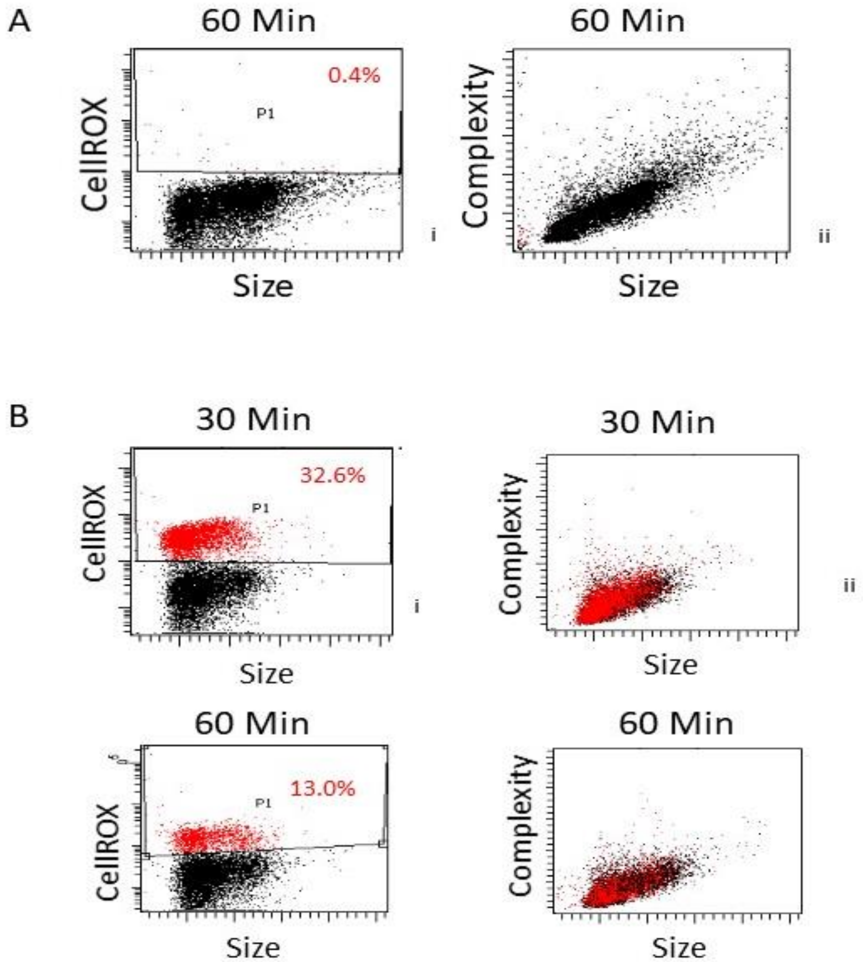

Figure 8. ***A temporal determination of the immediate, generation of ROS from yeast cells following acute treatment with cadmium.*** *Representative data of cytometric analysis of CellRox deep red dye oxidation in cells treated with (A) 50 µM PO_4 as a control, (B) 8 mM copper nitrate for 30 min and (C) 8 mM copper nitrate for 60 min. For each set of cells, cytometric evaluations included a comparison of CellRox dye oxidation vs cell size(forward scatter) - denoted bye numerals 'i', and an analysis of the same population of cells comparing complexity (side scatter) vs forward scatter (cell size) denoted by numerals 'ii'.*

3.3 Acute heavy metal treatment causes apoptosis in *S. cerevisiae*

Caspase activation was analyzed using SR-FLICA (VADFMK) a fluorescent probe that specifically binds to activated caspase 2 or 7, and which has also been shown to bind the singular yeast caspase (Nargund, 2014). Following the addition of copper and cadmium for one hour, the metal exposed cells were assessed for the presence of activated caspase, following a three-hour refractory period, in which sufficient caspase could be activated to bind the FLICA dye. Background levels of activated caspase were shown in the control group, which had not been exposed to any metal, and the population was centered around quadrant three and four, with a 'halo' of larger cells extending into quadrant two; there was a small population of cells jutting from the lower-left portion of the main population in quadrant three in the size vs complexity graph (Fig. 9A). Activation of caspases in cells which were treated with 80 μM cadmium for one-hour approximated 6% of the population, and corresponded to the smaller and less complex cells, as might be expected for either freshly budded cells or apoptotic cells, which very often shrink as they undergo programmed cell death (Fig. 9B). In addition, the flow cytometric scatter plots for all cadmium exposed cells produced dye bound signals (Fig. 9B, P2), which were much smaller than normal yeast cells, and may correspond to cellular debris or potentially membrane-bound 'blebs' that contained activated yeast caspase. In contrast, cells exposed to 8 mM copper (II) for one hour produced a subpopulation of cells that appeared to produce activated caspase, which constituted up to 28.0% of the total cell population; and which again appeared to be smaller in size than the average yeast cell (Fig. 9C).

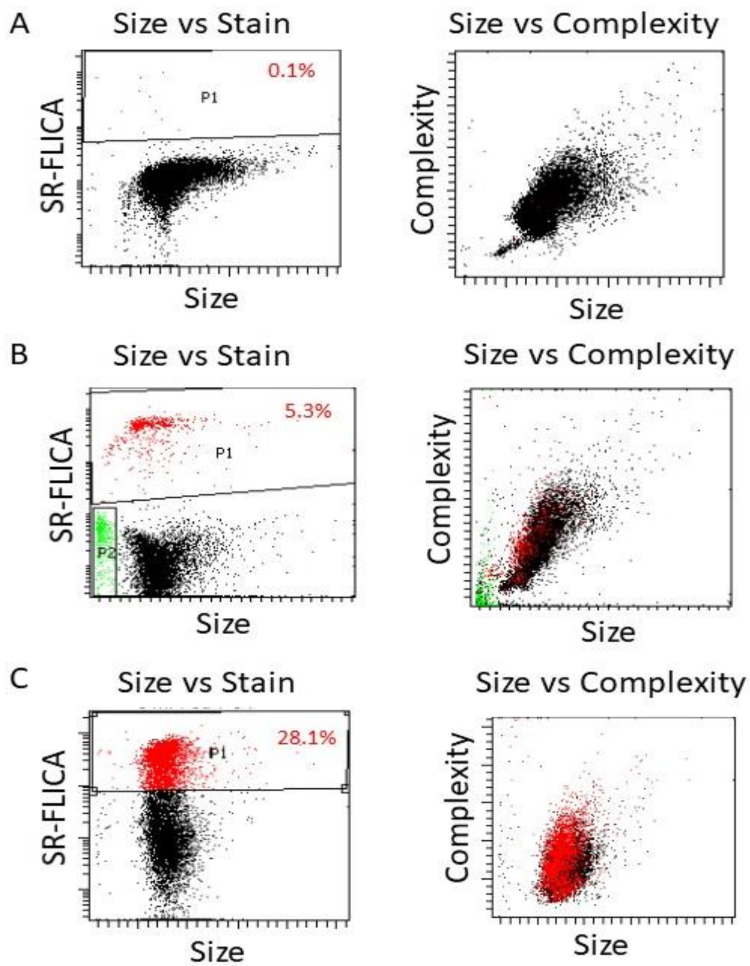

Figure 9. Evaluation of activated yeast caspase following acute exposure (1-hr) to the heavy metals, cadmium and copper. *Acute heavy metal treatment with copper (II) nitrate or cadmium nitrate causes caspase-dependent cell death in S. cerevisiae. Representative data of cells grown to mid-log phase and treated for 1-hour with either (A) 50 µM PO_4 as a control, (B) 80 µM $Cd(NO_3)_2$, or (C) 8 mM $Cu(NO_3)_2$. For each set of cells cytometric evaluations included a comparison of SR-FLICA (VADFMK) poly-caspase detector vs cell size (forward scatter) and an analysis of the same population of cells comparing complexity (side scatter) vs cell size (forward scatter). Red numbers indicate populations undergoing apoptosis. Population P2 in (B) was subtracted from total counts to calculate apoptosis in cadmium treatment. Subpopulations of cells that possess activated caspases which bind to SR-FLICA were denoted by % value of the total population in all cases.*

Even though Propidium iodide is often used as a dye to demonstrate necrosis in mammalian cells, it has been shown in our laboratory that PI staining correlates well with cells that have activated caspase and will eventually undertake acute, metal-induced apoptosis, at least for cells exposed to cadmium (Nargund, 2014). This apparently yeast specific effect was confirmed when cells which had been exposed to cadmium for only one hour were labelled with both PI and the green fluorophore, FAM-FLICA (DEVD), (Fig. 10). Background levels of FAM-FLICA and PI stained cells were at levels similar to control groups for previous assays with SR-FLICA and PI (Fig. 10A). In the cells treated with cadmium for one hour, however, the caspase-specific FLICA dye stained 6%, the majority of which were also stained with PI. There was a small population of cells in the lower-right quadrant, approximately 23% of all the FLICA stained cells, that reflected a subpopulation of cells that possessed high levels of activated caspases, but which were unable to bind PI (Fig 10B). In cells that had been similarly treated with copper, the subpopulation of cells with activated caspase (FAM-FLICA) stained cells was about 25%, with a similarly sized proportion of this subpopulation (~21%) failing to bind PI. In addition, a third subpopulation appeared (approximately 4% of the total population) which bound PI but failed to be stained with FAM-FLICA (Fig. 10C).

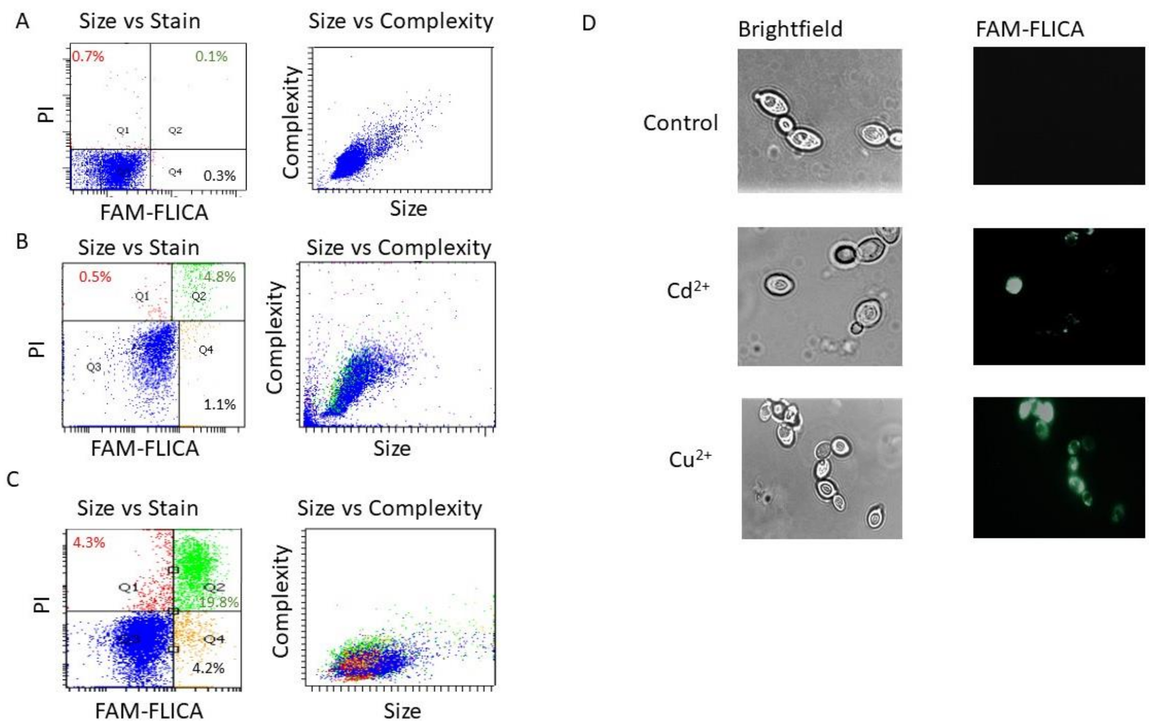

Figure 10. Analysis of correlation between FAM-FLICA stained cells and PI stained cells following 1-hour treatment with heavy metals cadmium and copper in S. cerevisiae. *There is a tight correlation between FAM-FLICA staining following one-hour treatment with cadmium, while copper treatment gives rise to disparate populations of PI and FAM-FLICA stained cells. Representative data of cytometric analysis of activated caspases utilizing FAM-FLICA (DEVD) in yeast cells treated with (A) 50 µM of* PO_4 *as a control, (B) 80 µM* $Cd(NO_3)_2$ *, or (C) 8 mM* $Cu(NO_3)_2$*. For each set of cells, cytometric evaluations include a comparison of SR-FLICA intensity vs PI intensity and an analysis of the same cells for complexity (side scatter) vs cell size (forward scatter). In all cases, dead/dying cells are reported as percent of total population. Red numbers indicate PI stained cell population, green numbers indicate dual stained population, and purple numbers indicate FAM-FLICA stained population percentages. Panel D is the visualization of caspase activation with the fluorophore FAM-FLICA using a Zeiss microscope, original magnification 40x.*

3.4 The effect of cadmium and copper exposure on glutathione biosynthesis after one-hour treatment in *S. cerevisiae*

As discussed previously, glutathione is one of the central mechanisms by which cells mitigate damage that may result from exposure to specific metals and ROS. The presence of glutathione in the cell was assayed using a GHS:GSSG colorimetric assay, which utilizes Ellman's reaction with free thiols in glutathione. Figure 11 depicts the relative amounts of glutathione, GSH and GSSG within the cells that have been exposed to either cadmium or copper for one hour when compared with cells that had been exposed to neither. In the control group, total glutathione was about 40 nM x $cell^{-1}$, oxidized glutathione approximating 0.6 nM x $cell^{-1}$; giving a GSH/GSSG ratio approximating 62. In contrast, exposure of cells to cadmium gave rise to a significant increase in total glutathione raising it to level approximating 140 nM x $cell^{-1}$. With GSSG levels in cadmium exposed cells at or around 3 nM x $cell^{-1}$, the GSH/GSSG ratio remained similar to that of the untreated cells at approximately 57. In cells treated with the direct-ROS generating copper (II), total glutathione level remained about 40 nM x $cell^{-1}$, but levels of oxidized glutathione were increased to 3 nM x $cell^{-1}$, resulting in a GSH/GSSG ratio that is 15 (Fig. 11B), indicating a dramatic increase in 'oxidative stress' within these copper treated cells.

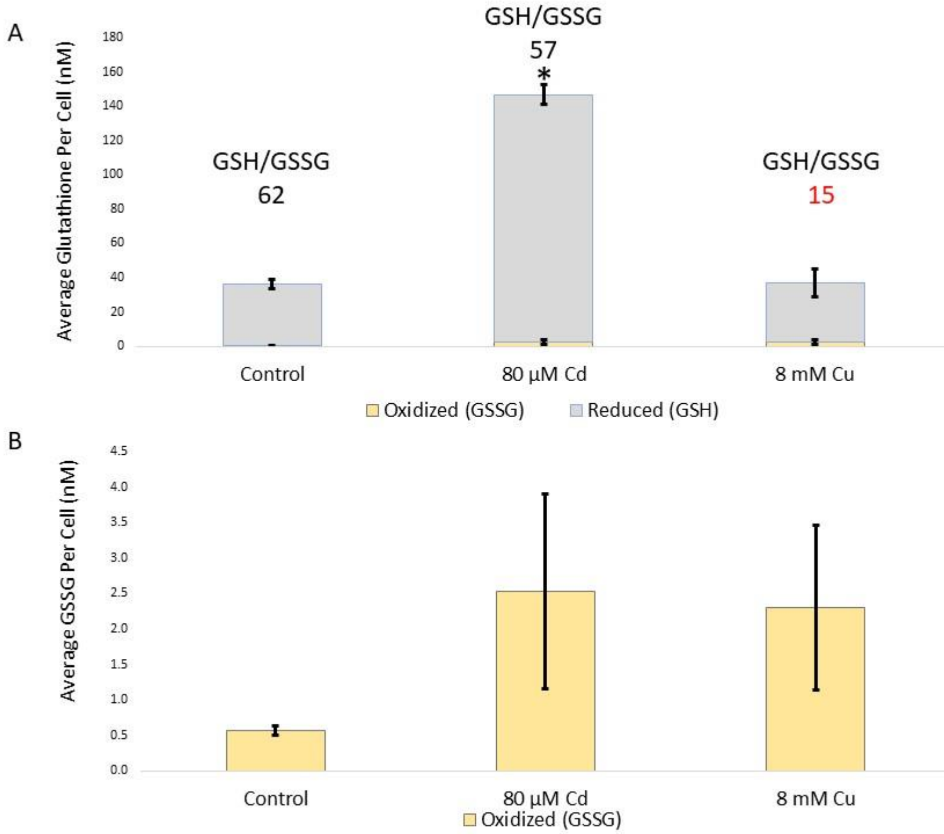

Figure 11. Assessment of intracellular glutathione levels in yeast following acute exposure (1 hr.) to cadmium or copper. *Glutathione levels are reported for (A) total and (B) oxidized glutathione utilizing an Enzolifesciences GSH:GSSG detection kit. GSH/GSSG ratios are denoted above in (A); black numbers indicate GSH/GSSG ratios near or at basal levels of oxidative stress under no stress condition and red numbers indicate cells experiencing high oxidative stress. Reported values are an average of three independent experiments. Comparison between control and treatment groups denoted with * indicates p<.05 (t-test, n=6).*

3.5 Investigation of FFAAPs supplementation on ROS generation in *S. cerevisiae*

As indicated in the introduction section of this study, it is anticipated that the addition of FFAAPs to the growth media ought to provide critical precursors to glutathione for the cells, which may confer a resource for the cells to offset some of the more harmful effects of metal-induced stress that result even from an hour of exposure to low-levels of metals. Thus, the second portion of these studies was designed to investigate the cellular responses of heavy-metal treated yeast grown in media supplemented with the FFAAPs necessary for glutathione biosynthesis. Supplemental FFAAPs was provided to cells during the culture phase at a concentration of 2 mM. Intracellular ROS generation was analyzed using CellROX deep red fluorescent probes. After 60 minutes of treatment with untreated cells grown in a buffered media containing 2 mM FFAAPS the levels of ROS, as determined by the binding of CellROX deep red dye, presented a consistently low background level, approximating 0.3% (Figs. 12A). One-hour exposure of these cells to 80 µM of cadmium for 30 min. or 60 min. failed to raise the levels of ROS above background levels (Fig. 12B).

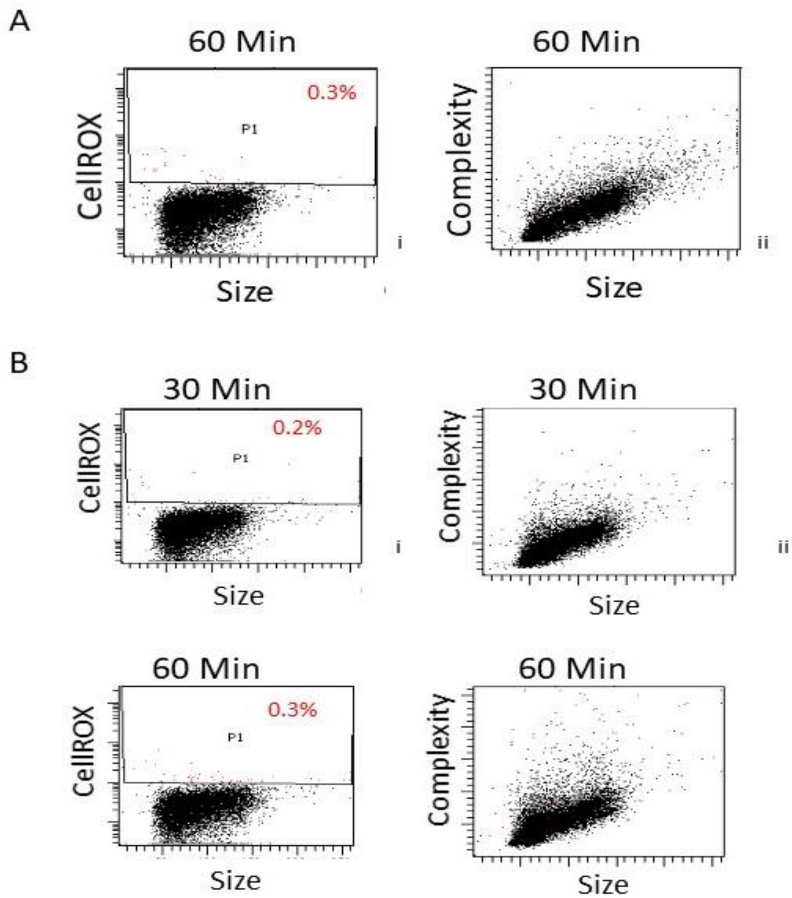

Figure 12. Temporal determination of immediate generation of ROS from yeast cells cultured in FFAAP following acute (1-hr) treatment with cadmium. *Acute cadmium treatment produces populations of cells with ROS generation at background levels for S. cerevisiae cultured in FFAAPs containing media. Representative data of cells that were treated with (A) 50 µM* PO_4 *or (B) 80 µM cadmium nitrate for one hour. For each set of cells, cytometric evaluations included a comparison of CellROX dye vs cell size (forward scatter) -denoted by numerals 'i', and analysis of the same population of cells comparing cell size (forward scatter) vs complexity (side scatter) -denoted by numerals 'ii'. In all cases, ROS levels were denoted by % values of the total population.*

Exposure of cells grown in the presence of FFAAP to 8 mM copper for 30 min, however, resulted in the generation of a significant subpopulation of these cells (~40%) to possess heightened levels of ROS, as measured by CellROX (Fig. 13B). This subpopulation with high ROS levels dropped significantly to 9.4% of cells after 60 min., indicating that the cells were able to mount a significant response to copper exposure, even within 1 h.

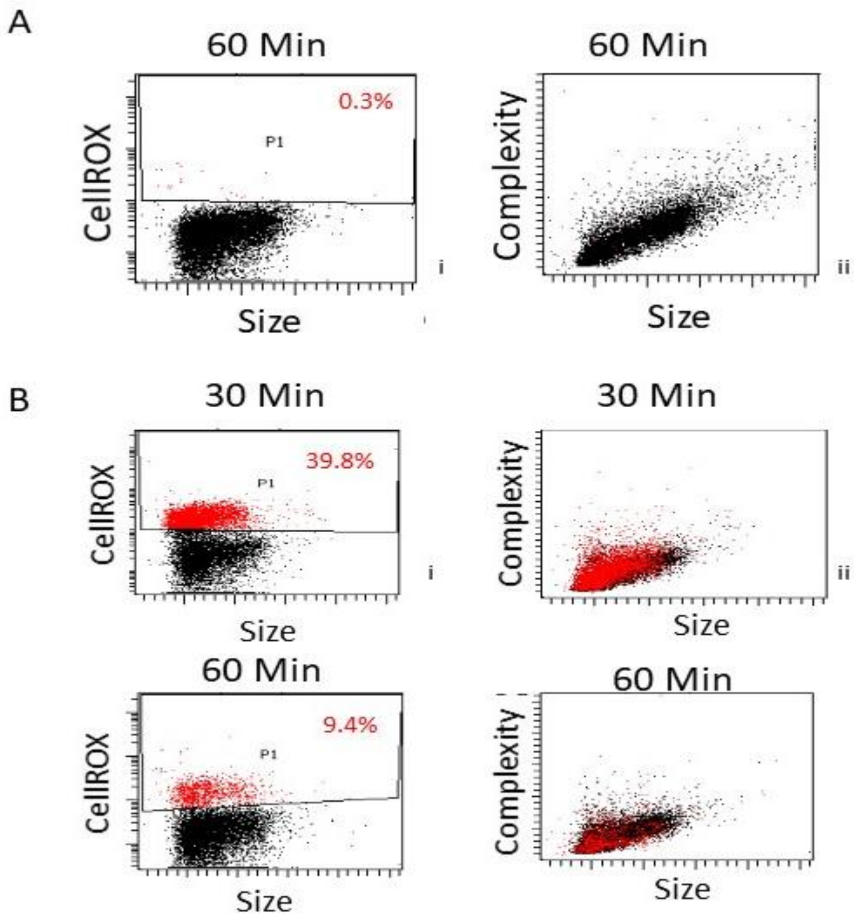

Figure 13. Temporal determination of immediate generation of ROS from yeast cells cultured in FFAAP following acute (1-hr) treatment with copper. *Acute copper treatment produces populations of cells with ROS generation considerably above background in S. cerevisiae cultured in FFAAPs containing media. Representative data of cells that were treated with (A) 50 µM PO_4 or (B) 80 µM copper nitrate for one hour. For each set of cells, cytometric evaluations included a comparison of CellROX dye vs cell size (forward scatter) -denoted by numerals 'i' and analysis of the same population of cells comparing cell size (forward scatter) vs complexity (side scatter) -denoted by numerals 'ii'. In all cases, ROS levels were denoted by % values of the total population.*

3.6 Effect of FFAAPs on apoptotic levels in *S. cerevisiae*

To determine if FFAAP treatment was able to cause any effect on metal-induced programmed cell death (PCD), apoptotic studies were undertaken using first SR-FLICA (Fig. 14) and then, again with FAM-FLICA together with PI (Fig. 15). For cells stained with the red fluorophore SR-FLICA, control cells presented background levels of apoptosis (Fig. 14A). Cells which were treated with 80 µM $Cd(NO_3)_2$ for one hour gave rise to a subpopulation (~4.8%) of cells that possessed sufficiently high levels of activated yeast caspase to promote apoptosis. As in Figure 9, exposure of the cells to cadmium did elicit a subpopulation of exceedingly small, sub-cellular sized particles (P2) that did not appear to bind SR-FLICA well (Fig. 14B). About 30% of the cells treated with 8 mM $Cu(NO_3)_2$ did demonstrate some apoptotic effects (Fig. 14C). Size dynamics of this subpopulation would again suggest this subpopulation was compromised of relatively smaller cells when compared to the overall population of untreated cells.

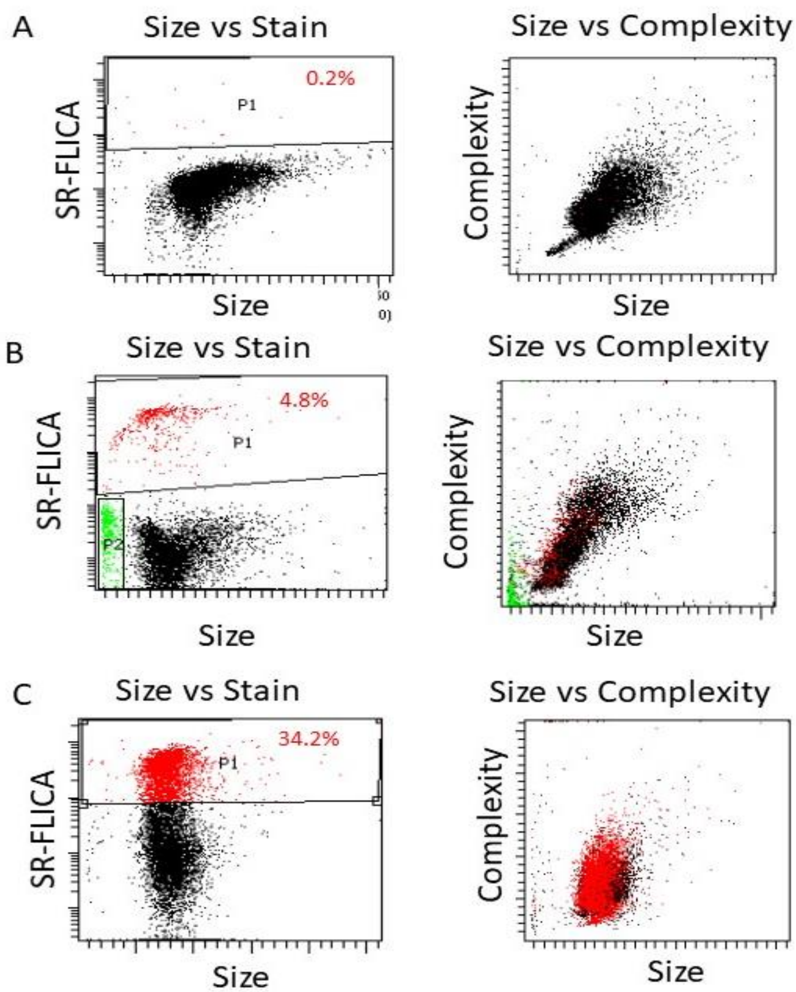

Figure 14. Supplemental FFAAPs treatment induces a slight reduction in the population size of cells undergoing apoptosis following acute heavy metal treatment for 1h. *Representative data of cells which were grown to mid-log phase in FFAAPs media and treated for 1-hour with either (A) 50 µL PO_4, (B) 80 µM $Cd(NO_3)_2$, or (C) 8 mM $Cu(NO_3)_2$. Caspase activation was decreased after acute cadmium treatment and slightly increased after acute copper treatment, relative to cells cultured in non-FFAAPs media. In all instances, caspase activation was reported as % total population.*

To demonstrate that cells that bind PI approximate those cells that represent caspase-activated yeast cells when exposed to 8 mM copper, dual staining with FAM-FLICA and PI was undertaken (Fig. 15). The control group in FFAAPs had FAM-FLICA staining slightly higher than other control groups at about 1% (Fig. 15A). PI staining was at background levels for cells treated with cadmium for one hour, and caspase activation was seen to occur in about 5% of the population (Fig. 15B). In the group treated with 8 mM copper, about 19% of the cells had activated caspase, and the population of PI stained cells was about 8% (Fig. 15C). General cell size dynamics were similar to previous studies, with a slight overall decrease in total cell volume and cellular inclusions for both metal treatments.

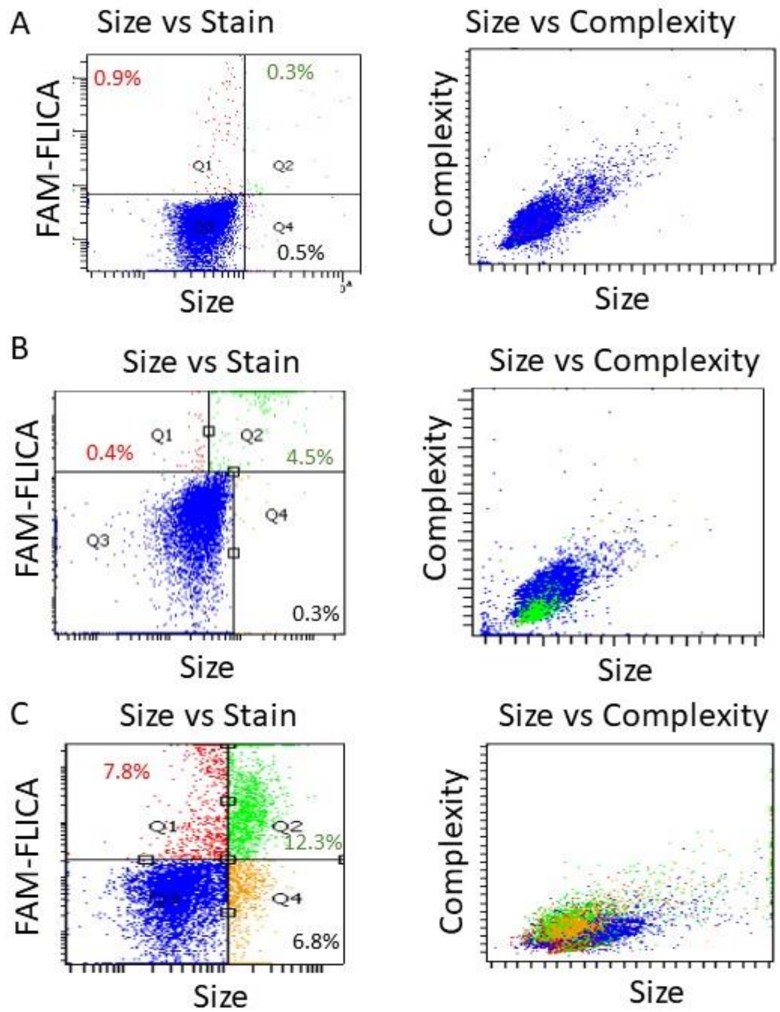

Figure 15. Analysis of PI as a marker for apoptosis in yeast supplemented with FFAAPs following 1-hour treatment with heavy metals cadmium and copper in S. cerevisiae*. There was a tight correlation between FAM-FLICA and PI following cadmium treatment in yeast, while copper treatment gives rise to disparate populations of cells stained with FAM-FLICA and PI. Representative data of cytometric analysis of activated caspases utilizing FAM-FLICA (DEVD) in yeast cells treated with (A) 50 µM of PO_4 as a control, (B) 80 µM $Cd(NO_3)_2$, or (C) 8 mM $Cu(NO_3)_2$. For each set of cells, cytometric evaluations include a comparison of SR-FLICA vs PI and an analysis of complexity (side scatter) vs cell size (forward scatter). In all cases, dead cells are reported as percent of total population. Red numbers indicate PI stained cell population, green numbers indicate dual stained population, and black numbers indicate FAM-FLICA stained population percentages.*

3.7 Glutathione biosynthesis for *S. cerevisiae* cultured in FFAAPs media

To determine the effects of FFAAPs supplementation for glutathione biosynthesis, Ellman's reaction was again employed, as before. The synthesis of glutathione in yeast grown in media containing 2 mM FFAAPs was assayed using Enzolifesciences GSH:GSSG detection kit under otherwise identical conditions to those referenced in Figure 11. The levels of GSH and GSSG in yeast treated with PO_4, cadmium, and copper for one hour were averaged from three independent assays and displayed (Fig 16). For the control cells, there was about 22 nM x cell^{-1} total glutathione and about 0.3 nM x cell^{-1} GSSG; the GSH/GSSG ratio for these cells was about 91. The cadmium treatment group had a total glutathione concentration of around 60 nM x cell^{-1} and 0.4 nM x cell^{-1} of GSSG; with a GSH/GSSG ratio of 135. Copper treated cells were analyzed to contain about 27 nM x cell^{-1} total GSH, and about 0.5 nM x cell^{-1}, and a GSH/GSSG ratio approximating 61 (Fig 16A & B). Figure 16B was included to more clearly display oxidized levels of glutathione.

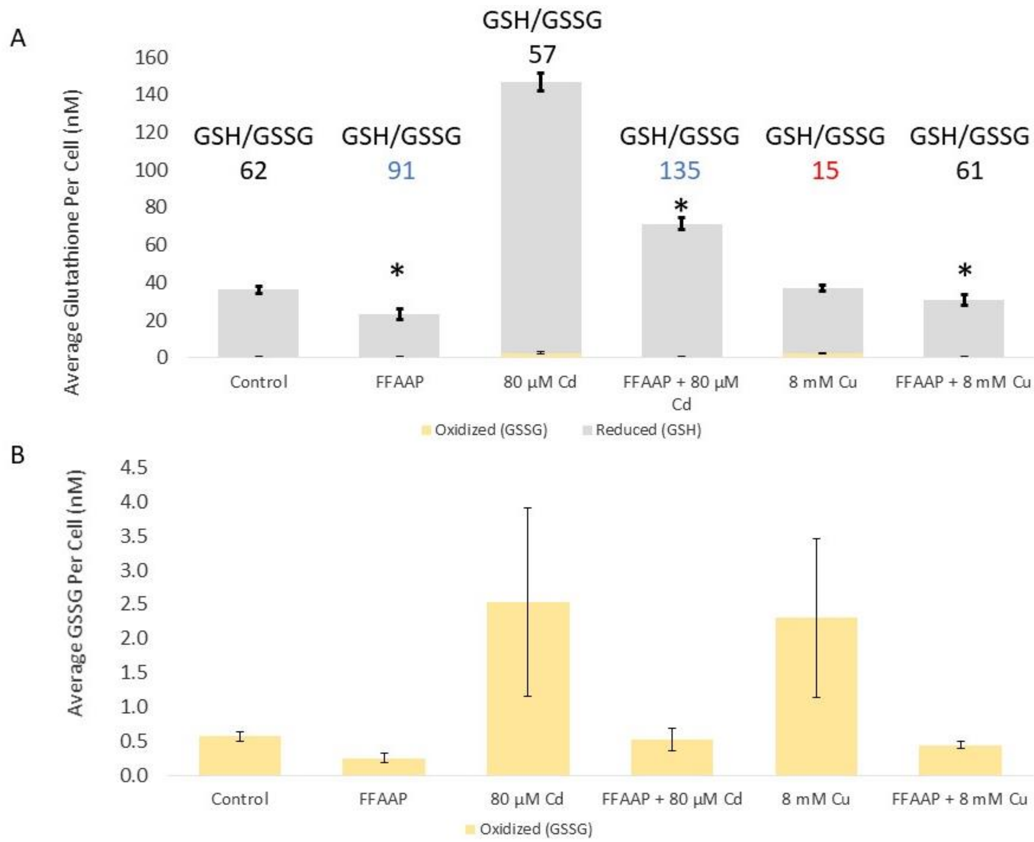

Figure 16. FFAAP supplementation changes glutathione biosynthesis in S. cerevisiae following cadmium or copper treatments of 1h. *Glutathione levels are reported for oxidized and reduced glutathione, as shown previously. GSH/GSSG ratios are denoted in (A); black numbers indicate GSH/GSSG ratios at or near basal, red numbers indicate cells with heightened oxidative stress, and blue numbers indicate cells with lowered oxidative stress. Reported values are the average of three biological replicants. Comparison between FFAAPs treatments and metal-treated groups denoted with * indicates p<.05 (T-test, n=11). FFAAPs treatment reduces both reduced and oxidized glutathione levels under metal conditions, relative to yeast that were not exposed to heavy metals and/or which were not recipients of the FFAAPs dietary supplement.*

4 Discussion

These investigations were designed to elucidate the underlying cellular mechanisms that *S. cerevisiae* employ in response to acute heavy metal toxicity, specifically cadmium and copper. Also, the question of how FFAAPs supplementation affected cell survivability via glutathione homeostasis was addressed. In addition, the question of whether and how FFAAPs supplementation affected these response mechanisms, potentially by providing an abundance of the amino acid precursors of glutathione, was also addressed. Previous work within the lab determined subtoxic concentrations for each of the metals by conducting growth curve studies under a series of increasing metal concentrations, with the highest concentration of metal which did not affect doubling time was chosen (Nargund, 2014; Creech *et al.* unpublished manuscript). The term "subtoxic" may be somewhat of a misnomer as these levels of metal exposure do induce a relatively small subpopulation of cells do initiate a caspase-specific apoptotic response in the longer term, see Fig. 9, without affecting the immediate growth dynamics.

Redox-inactive versus redox-active heavy metals

As more heavy metals are introduced into the food-web due to human activities, heavy metal intoxication is becoming a growing concern (Su, 2014), and the role of yeast served as a model system to understand these concerns increases accordingly. Cadmium and copper were chosen as they exemplify two different types of heavy metals, redox-inactive and redox-active, respectively. As exemplars of each type of heavy metal, both cadmium and copper provide two different types of cellular insults and associated cellular responses. Cadmium and copper caused heterogeneous cellular responses. Cadmium failed to produce increased ROS levels in any of the exposed yeast within the first hour of treatment, while copper caused a relatively large ROS response in a subpopulation of cells within the first 30 minutes followed by a decline in the subpopulation with high ROS levels after 60 minutes of treatment. In additions, both metals also caused different apoptotic phenotypes with copper giving rise to a larger subpopulation of cells showing apoptotic response. Also, cellular responses via the antioxidant properties arising from cycling of glutathione (GSH to GSSG) were different for each metal.

ROS accumulation due to heavy metal treatment

Increased levels of ROS within the cells has been implicated in population differentiation, cell death via apoptosis, and other mechanisms of cell death including necrosis (Buttke, 1994; Thayyullathil, Chathoth, Hago, Patel, & Galadari, 2008). Thus, intracellular ROS levels resulting from either redox-inactive or redox-active heavy metals were assessed using CellROX deep red fluorescence probe. Cadmium did not increase ROS levels above the untreated group in the first hour of treatment (Fig. 7), while copper caused a dramatic increase over the untreated group in the first 30 minutes; the ROS subpopulation greatly decreased under copper treatment after 60 minutes of copper treatment indicating the cells have initiated processes for returning to oxidative homeostasis (Figs. 8).

Previous researchers within this lab already discovered that the changes in expression profile begin within 15 minutes of heavy-metal treatment (Nargund, 2014); Thus, it is evident that copper treated yeast initiate ROS protective pathways before 60 minutes of exposure. Also, the size of the cells in the subpopulation demonstrating high ROS levels generally became smaller with both copper and cadmium treatment which is indicative of yeast under cytotoxic stress as noted during these investigations, and potentially indicates that the process of apoptosis has initiated. Cells undergoing apoptosis will shrink and ‘bleb’ into small fragments sequestering toxic elements (Farrugia & Balzan, 2012). Even though cadmium did not induce ROS within the first hour of treatment, a small subpopulation of yeast induced apoptotic pathways after a single hour of treatment.

Apoptotic response due to acute heavy metal treatment

Investigation of apoptosis following one-hour treatment of the redox-inactive metal cadmium versus the redox-active metal copper proceeded next. SR-FLICA was initially used to investigate this phenomenon (Fig. 9). The apoptotic population within the cadmium group was at levels previously seen in the lab, and copper-induced apoptotic response in a subpopulation that possessed more individuals than copper. It has been discovered that expression of the single yeast caspase, YCA1, increase 2-fold by five minutes post-treatment which is sustained for the duration of cadmium treatment (Nargund, Avery, & Houghton, 2008). Because of spectra overlap, PI cannot be concomitantly stained with SR-FLICA. Thus, it was decided to conduct the caspase-dependent cell death assay with FAM-FLICA, a green fluorophore so that dual staining could proceed with PI.

Analysis of the cadmium treated group dual stained with FAM-FLICA and PI showed similar levels of caspase-activated yeast relative to SR-FLICA assays and caspase-independent cell death was at background (Figs. 9B & 10B). The cell death associated with acute cadmium treatment must be completely caspase-dependent, as a population of cells in the PI only quadrant were at background. The fact that cadmium treatment activates caspase within the first hour of treatment while unaffecting ROS levels suggest atypical ROS-independent apoptosis initiation (Yokouchi, *et al.,* 2007). It was found that after three hours, cadmium does produce a subpopulation of yeast with increase ROS levels and damaged mitochondria (Nargund, 2014). Acute copper treatment activates caspases as well as initiates caspase-independent cell death as there was less correlation between caspase activation and cell death in acute copper treatment;

indicating that the cellular damage resulting from cadmium treatment is more focused than damage resulting from copper treatment. Variability in FLICA staining versus PI in the copper group may be a result of generic damage arising from ROS due to copper treatment as well as copper specific induced mechanisms (Fig. 3); thus, the response resulting from copper treatment is multifaceted while cadmium-induced apoptosis appears more discrete. Microscopy further demonstrates that metal treatments affect only a subpopulation of the cells treated with ‘subtoxic’ levels of heavy metals; the white portion in microscopy are a result of over-exposure.

Glutathione response after acute heavy metal treatment

Glutathione is both a central defense against intracellular ROS as well as discrete metal cations (Rubino, 2015). The antioxidant properties of glutathione require cycling between reduced GSH and oxidized GSSG with the aid of enzymes and $NAPDH_2$ (Inoue *et al.,* 1999). Comparison of glutathione levels in *S. cerevisiae* as a result of cadmium and copper exposure was enabled by the use of a sensitive assay that is able to differentiate between the intracellular concentrations of GSH and GSSG (Enzolifesciences). The results for total glutathione demonstrated there was no change in the overall glutathione levels in copper treated group for total glutathione, but the cadmium treated group had a significant increase in total glutathione of over 400% ($P<.05$, $n=6$), (Fig. 11A). The contrast in glutathione levels was somewhat surprising in light of how tightly glutathione pools are regulated in *S. cerevisiae* and in higher eukaryotes. The striking change in glutathione levels in the presence of cadmium, even after only one hour of exposure, obviates a transcriptional increase that has been suggested in this lab and elsewhere (Vido, 2001; Creech *et al.* unpublished observations) and has prompted a targeted transcriptomic analysis that is beyond the bounds of this study, but which remains an immediate focus of our research (see appendix A). It is also likely that cadmium causes increased glutathione levels because cadmium is one of the metals cations that specifically binds with GSH and which, thereafter is sequestered into vacuoles, thus decreasing cytosolic pools of glutathione (Li *et al*., 1997). Thus, potentially a portion of the increase in total glutathione could be due to this immediate recycling of GSH from the GS-Cd-SG conjugates, which are known to arise under acute cadmium stress in yeast.

In contrast, copper-induced stress does not appear to induce any glutathione biosynthesis. Interestingly, even when all the genes responsible for the synthesis of glutathione from its constituent amino acids were fully induced, as they have been (Ask *et al.,* 2013), total glutathione pools only increased ~40%. Thus, for cadmium to initiate >400% increase in total glutathione, the response of the cell would appear to be far more complex than simple transcriptional induction of a few genes. Curiously, even this large increase in total glutathione pools.

The levels of GSSG, following 1 h. cadmium treatment, were similar to levels which arise as a result of acute copper treatment (Fig. 11B), even though intracellular ROS levels remained at background levels after acute cadmium treatment. A mechanism whereby glutamine-metal conjugates can competitively inhibit glutathione reductase cycling has been proposed, which would reduce the effectiveness of this enzyme to recycle GSSG into two GSH molecules (Rubino, 2015). Natural processes in the cell would induce GSSG production, and reduced efficacy of glutathione reductase due to increased levels of glutathione-conjugated cadmium would retard cycling back to GSH in cadmium treated *S. cerevisiae* until GSSG concentrations reach levels sufficient to outcompete the inhibition.

The similarity in levels of oxidized glutathione between redox-active and -inactive metals indicates that it is appropriate to use the ration of GSH/GSSG to provide a suitable comparison among and between the effects that acute exposure to copper and cadmium and copper on the redox reactions undertaken by glutathione. A decrease in the GSH/GSSG ratio would indicate a shift in glutathione levels toward GSSG, indicating the cells are under oxidative stress, while an increase in the ratio would indicate a shift toward reduced glutathione (GSH) corresponding to lowered cellular oxidative stress (Ansesi *et al.*, 1999). Figure 16 demonstrates these changes and how they relate to the oxidatively stressed state of the cells under different conditions. The untreated group had the highest ratio of 62, cadmium was relatively unchanged from this control with 57, and copper demonstrated the greatest shift toward GSSG with a ratio of 15, confirming the highly oxidatively stressed state of these particular cells (Fig. 8). There must be a mechanism, therefore, whereby yeast sense the GSH/GSSG ratio and attempt to rebalance the ratio back to basal levels. Chakravarthi proposed**, that in mammalian endoplasmic reticulum (ER), the balance is maintained by the formation and/or breaking of disulfide bridges using GSSG or GSH respectively, through the activity of protein disulfide isomerase. Our results would support such a proposal; however, more work needs to be done to expand this model to total yeast GSH/GSSG ratios.

The role of FFAAPs in cellular responses to acute heavy metal treatment

The second portion of this overall study was also to assess the differences in cellular processes for yeast treated in the presence of Free Form Amino Acid Precursor (FFAAPs) supplements. We are the first to assess the effects of FFAAPs on yeast under acute heavy metal treatment, and the results are intriguing. FFAAPs are supplemental additions of necessary amino acids for non-ribosomal produced pseudo peptides. The supposition is that the addition of appropriate amino acids will shift steady-state equilibrium of glutathione biosynthesis towards the product (GSH). As depicted in Fig. 16, the presence of FFAAPs caused a significant decrease in total glutathione pools under all conditions tested, even within the control cells. Moreover, the GSH/GSSG ratios were found to increase under all conditions tested. While the latter finding is consistent with the expected role of FFAAP supplements, and the fact that they caused this increase in ratios and at the same time lowered the total glutathione pools is surprising.

Glutathione levels as a result of FFAAPs supplementation

The decrease in total glutathione levels brought upon by FFAAPs is no less significant than in cells treated with cadmium. Even when the production of glutathione is so dramatically enhanced by the presence of the metal, the presence of FFAAPs caused a 50% reduction in the levels of glutathione, as well as a concomitant dramatic increase in GSH/GSSG ratios from basal levels (~60) to substantially reduce levels of 135.

The decrease in GSSG (Fig. 16B) levels associated with FFAAPs treatment for all conditions tested can be explained, at least in part, by lowered oxidative stress brought on by a reduction in levels of oxidized glutathione. Also, the GSH/GSSG ratio increased in all FFAAPs media groups, including the untreated group, by 0.5- to 4-fold relative to cells of the same treatment type without FFAAPs media, which is indicative of a change to a more “robustly” maintained reduced state inside the cell. These findings indicate that FFAAP treatment induces cellular programs to return the GSH/GSSG ratio to basal levels or higher, especially under heavy metal assault; thus, resetting glutathione homeostasis. Furthermore, the finding that FFAAPs actually increases glutathione levels by about 20% in mammalian cells (Vasireddi *et al.*, 2019) but not in yeast suggests that *S. cerevisiae* may provide a sensitive sensor for oxidative stress.

The reducing power of glutathione is dependent upon GSH concentrations available for redox reactions and pools of cytosolic $NADPH_2$ (Inoue *et al.*, 1999). Cellular GSH levels increase with the synthesis of new GSH or by recycling GSSG into two molecules of GSH (Noctor, 1998; Diaz-Vivancos, Wolff, Markovic, Pallardó, & Foyer, 2010). Thus, the faster GSSG cycles to GSH, the better a cell can potentially protect against ROS damage and/or cadmium intoxication.

The effects of FFAAPs on ROS generation after acute heavy metal treatment

CellRox deep red oxidative probe was used to determine cytosolic ROS levels of yeast cultured in FFAAPs and then treated with various heavy metals. There was nominal ROS generation within one hour of treatment with cadmium relative to untreated groups, and copper increased ROS generation greatly (Fig. 13). In the copper group, the population displaying high ROS levels was greatest 30 minutes post-treatment, followed by a sharp decline in the population with high ROS levels at the 60-minute time point (Fig. 13B). One possible conclusion is that the apparent recovery of cells from high ROS levels in the second 30 minutes of exposure successfully reduced the ROS levels by initiating cellular programs, including the recycling of glutathione pools for oxidative stress protection. Oxidative homeostasis is a delicate balance between ROS and antioxidants, and a cell will attempt to maintain this balance (Foyer, & Noctor 2005). Generally, the ROS subpopulations treated with copper for 1 h. were higher in FFAAPs cultured group when compared to non-FFAAPs cultured cells at the 30 minutes, but slightly lower at the 60-minute time point. Initial increases in ROS levels in subpopulations with FFAAP indicate a cost associated with decreased glutathione pools resulting from the presence of FFAAPs. While the cells experience a reduction in oxidative stress (as expressed by the GSH/GSSG ratio [Fig. 16A]), reduced total glutathione levels effectively decrease the robustness of any subsequent antioxidant response to any future insult. Even so, the combined results of decreased levels of ROS in subpopulations of cells along with increased GSH/GSSG ratio indicate that FFAAPs does provide additional protection against oxidative stress during acute cadmium and copper treatment for *S. cerevisiae* irrespective of the initial cost associated with lowered glutathione pools.

The effects of FFAAPs supplementation on apoptosis

One of the initial aims of this study was to determine if FFAAPs treatment would increase cell survivability following acute heavy metal treatment. To investigate this, FAM-FLICA and SR-FLICA were used to determine activation of the singular yeast caspase apoptotic response, resulting from heavy metal treatment. As depicted in Figs 14B and 15B, there was ~17% decrease in caspase activation for acute cadmium treatment, resulting from the addition of FFAAPs. However, the results for copper treated cells were more ambiguous (Fig. 14C). The reduction in apoptosis for cadmium exposed cells provides a discernable phenotype that can be potentially be used for detecting the effects of FFAAPs supplement on *S. cerevisiae* under acute cadmium stress conditions. The inability of FFAAPs to have a similar effect upon copper treated cells may have something to do with the increased complexity of the cellular stress response in copper treated cells (with and without FFAAPs) than those treated with cadmium (Figs. 10B and 15B vs 10C and 15C).

FFAAPs supplementation potentially causes the cells to run genetic programs of low stress, possibly initialized by the change in the GSH/GSSG ratio. Upon introduction of a stressor, the reduced glutathione pools, due to resetting of glutathione homeostasis, decreases initial robustness of the cell to manage the assault. While we have no direct evidence, given the long-term sustainability of the more reduced glutathione steady-state, it is likely that FFAAPs treatment may be a better-suited treatment for chronic heavy metal stress in *S. cerevisiae*.

Future directions

A portion of this investigation initially involved undertaking a transcriptional analysis utilizing a custom “Nanostring panel” of pertinent genes responsible for expressing many of the proteins/enzymes that we have demonstrated are involved in yeast responses to heavy metal exposure (Creech, manuscript in preparation). However, a poorly timed malfunction of the instrument removed this option from being included in this study. Even so, a full list of genes in the panel is in Appendix A. Genes important to this study include genes involved in glycolysis including the pentose-phosphate pathway (PPP) defined previously in the lab when it was discovered that glycolytic proteins are strongly favored as targets of oxidation, and glutathione cycling requires NADPH of which the bulk arises from activation of the PPP (Nargund, 2014; Larochelle, Drouin, Robert, & Turcotte, 2006). Genes related to glutathione and cysteine biosynthesis were included in this panel, as their gene products have been directly implicated in glutathione levels (Noctor, 1998). Finally, metal-binding proteins were included genes, as it is known that metal-binding proteins are an important defense mechanism for the mitigation of heavy metal treatment in mammalian cells (Andrews, 2000). Other genes were also included in the panel to allow for investigation of related, but similar questions.

Further future directions for this study will focus on teasing out the control mechanism(s) on glutathione homeostasis through a series of molecular techniques on a series of pathway mutants to investigate phosphorylation levels of γ-ECS and transcription factors involved in cysteine synthesis, glycolysis, and PPP, or glutathione synthetase. Enzymatic studies of FFAAPs supplemented activities of γ-ECS and GS should also be considered. Such studies should go some of the way to develop a more detailed model identifying how yeast is able to accommodate exposure to different heavy metals, and perhaps be used as a sensitive sensor for oxidative pressure.

5 Conclusion

There was no induction of ROS in cells treated with cadmium for one hour relative to untreated cells, while there was about a 33% increase in ROS levels for cells treated with copper in the first 30 minutes, with a precipitous drop in ROS levels to about 14% at the 60-minute time point. Copper and cadmium both increase cell caspase-dependent apoptosis within one-hour of treatment, and there was a moderate increase in apoptosis for the copper treated group supplemented with FFAAPs. Cadmium activates the single yeast caspase within the first hour of metal treatment in a ROS-independent pathway, while avoiding caspase-independent cell death in yeast. Total glutathione levels decreased for cells grown in FFAAPs relative to cells grown in media lacking supplemental FFAAPs. Also, FFAAPs treatment allowed for a shift toward GSH, with the greatest shift occurring in the copper treated group, indicating that FFAAPs does reduce oxidative stress on all available groups, with the greatest amelioration of oxidative stress being in the redox-active copper. The oxidative stress yeast experience is directly related to the GSH/GSSG ratio, and glutathione-synthesis enzymatic dynamics are at least partially involved in the maintenance of said ratio.

REFERENCES

Andrews, G. K. (2000). Regulation of metallothionein gene expression by oxidative stress and metal ions. *Biochemical pharmacology*, *59*(1), 95-104.

Asensi, M., Sastre, J., Pallardo, F. V., Lloret, A., Lehner, M., Garcia-De-La Asuncion, J., & Viña, J. (1999). [23] Ratio of reduced to oxidized glutathione as indicator of oxidative stress status and DNA damage. In *Methods in enzymology* (Vol. 299, pp. 267-276). Academic Press.

Ask, M., Mapelli, V., Höck, H., Olsson, L., & Bettiga, M. (2013). Engineering glutathione biosynthesis of Saccharomyces cerevisiae increases robustness to inhibitors in pretreated lignocellulosic materials. *Microbial cell factories*, *12*(1), 87.

Bragado, P., Armesilla, A., Silva, A., & Porras, A. (2007). Apoptosis by cisplatin requires p53 mediated p38α MAPK activation through ROS generation. *Apoptosis*, *12*(9), 1733-1742.

Buttke, T. M., & Sandstrom, P. A. (1994). Oxidative stress as a mediator of apoptosis. *Immunology today*, *15*(7-10).

Cabiscol Català, E., Tamarit Sumalla, J., & Ros Salvador, J. (2000). Oxidative stress in bacteria and protein damage by reactive oxygen species. *International Microbiology*, *3*, 3-8.

Carmona-Gutierrez, D., Eisenberg, T., Buttner, S., Meisinger, C., Kroemer, G., & Madeo, F. (2010). Apoptosis in yeast: triggers, pathways, subroutines. *Cell Death Differ*, *17*(5), 763-773. doi:10.1038/cdd.2009.219

Chakravarthi, S., Jessop, C. E., & Bulleid, N. J. (2006). The role of glutathione in disulphide bond formation and endoplasmic-reticulum-generated oxidative stress. *EMBO reports*, *7*(3), 271-275.

Chen, C. Y., Stemberger, R. S., Klaue, B., Blum, J. D., Pickhardt, P. C., & Folt, C. L. . (2000). Accumulation of heavy metals in food web components across a gradient of lakes. *Limnology and Oceanography, 45*, 1525-1536.

Chen, E. Y., Fujinaga, M., & Giaccia, A. J. (1999). Hypoxic microenvironment within an embryo induces apoptosis and is essential for proper morphological development. *Teratology, 60*, 215-225.

Creech, M., Uei, D., Kaur, R., Valsan, S., Virdi, R., Liu, X., & Houghton, J. E. (unpublished). Proteome and Related Transcriptome Profiling of *S. cerevisiae* (Wild Type and YCA1Δ) in Response to Acute Exposure to Cadmium.

Crum, A. (2007). USA Patent No. 11/181,001. U.S. Patent Application.

Diaz-Vivancos, P., Wolff, T., Markovic, J., Pallardó, F. V., & Foyer, C. H. (2010). A nuclear glutathione cycle within the cell cycle. *Biochemical Journal*, *431*(2), 169-178.

El-Demerdash, F. M., Yousef, M. I., Kedwany, F. S., & Baghdadi, H. H. (2004). Cadmium-induced changes in lipid peroxidation, blood hematology, biochemical parameters and semen quality of male rats: protective role of vitamin E and beta-carotene. *Food Chem Toxicol, 42*(10), 1563-1571. doi:10.1016/j.fct.2004.05.001

Emerit, I. (1994). Reactive oxygen species, chromosome mutation, and cancer: possible role of clastogenic factors in carcinogenesis. *Free Radic Biol Med, 16*(1), 99-109. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/8300000

Evans, G. W. (1984). *Environmental stress*: CUP Archive.

Farrugia, G., & Balzan, R. (2012). Oxidative stress and programmed cell death in yeast. *Front Oncol, 2*, 64. doi:10.3389/fonc.2012.00064

Foyer, C. H., & Noctor, G. (2005). Redox homeostasis and antioxidant signaling: a metabolic interface between stress perception and physiological responses. *The Plant Cell*, *17*(7), 1866-1875.

Galan, A., Garcia-Bermejo, L., Troyano, A., Vilaboa, N. E., Fernandez, C., de Blas, E., & Aller, P. (2001). The role of intracellular oxidation in death induction (apoptosis and necrosis) in human promonocytic cells treated with stress inducers (cadmium, heat, X-rays). *Eur J Cell Biol, 80*(4), 312-320. doi:10.1078/0171-9335-00159

Gershon, H., & Gershon, D. (2000). The budding yeast, Saccharomyces cerevisiae, as a model for aging research: a critical review. *Mechanisms of ageing and development*, *120*(1-3), 1-22.

Goffeau, A., Barrell, B. G., Bussey, H., Davis, R. W., Dujon, B., Feldmann, H., . . . Oliver, S. G. (1996). Life with 6000 genes. *Science, 274*(5287), 546, 563-547. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/8849441

Inoue, Y., Matsuda, T., Sugiyama, K., Izawa, S., & Kimura, A. (1999). Genetic analysis of glutathione peroxidase in oxidative stress response of Saccharomyces cerevisiae. *J Biol Chem*, *274*(38), 27002-27009. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/10480913

Johnston, M. (1987). A model fungal gene regulatory mechanism: the GAL genes of Saccharomyces cerevisiae. *Microbiol Rev, 51*(4), 458-476. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/2830478

Kuan, C. Y., Yang, D. D., Roy, D. R. S., Davis, R. J., Rakic, P., & Flavell, R. A. (1999). The Jnk1 and Jnk2 protein kinases are required for regional specific apoptosis during early brain development. *Neuron, 22*, 667-676.

Larochelle, M., Drouin, S., Robert, F., & Turcotte, B. (2006). Oxidative stress-activated zinc cluster protein Stb5 has dual activator/repressor functions required for pentose phosphate pathway regulation and NADPH production. *Molecular and cellular biology*, *26*(17), 6690-6701.

Lawrence, R. A., & Burk, R. F. (1976). Glutathione peroxidase activity in selenium-deficient rat liver. *Biochem Biophys Res Commun*, *71*(4), 952-958. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/971321

Li, Z. S., Lu, Y. P., Zhen, R. G., Szczypka, M., Thiele, D. J., & Rea, P. A. (1997). A new pathway for vacuolar cadmium sequestration in Saccharomyces cerevisiae: YCF1-catalyzed transport of bis(glutathionato)cadmium. *Proc Natl Acad Sci U S A, 94*(1), 42-47. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/8990158

Lowe, S. W., & Lin, A. W. (2000). Apoptosis in cancer. *Carcinogenesis, 21*(3), 485-495. doi:10.1093/carcin/21.3.485

Lu, Z. H., Cobine, P., Dameron, C. T., & Solioz, M. (1999). How cells handle copper: a view from microbes. *The Journal of Trace Elements in Experimental Medicine, 12*, 347-360.

Marisa Repetto, J. S. a. A. B. (2011). Lipid Peroxidation: Chemical Mechanism, Biological Implications and Analytical Determination. *intech open*.

Mazzoni, C., & Falcone, C. (2008). Caspase-dependent apoptosis in yeast. *Biochim Biophys Acta, 1783*(7), 1320-1327. doi:10.1016/j.bbamcr.2008.02.015

McGary, K. L., Park, T. J., Woods, J. O., Cha, H. J., Wallingford, J. B., & Marcotte, E. M. (2010). Systematic discovery of nonobvious human disease models through orthologous phenotypes. *Proceedings of the National Academy of Sciences, 107*, 6544-6549.

Milne, L., Nicotera, P., Orrenius, S., & Burkitt, M. J. (1993). Effects of glutathione and chelating agents on copper-mediated DNA oxidation: pro-oxidant and antioxidant properties of glutathione. *Arch Biochem Biophys, 304*(1), 102-109. doi:10.1006/abbi.1993.1327

Nargund, A. M., Avery, S. V., & Houghton, J. E. (2008). Cadmium induces a heterogeneous and caspase-dependent apoptotic response in Saccharomyces cerevisiae. *Apoptosis*, *13*(6), 811-821.

Nargund, A. (2014). Analysis of Cellular Responses to Heavy Metal Induced Stress in Saccharomyces cerevisiae. PhD Dissertation. GSU. Atlanta, GA.

Noctor, G., Arisi, A. C. M., Jouanin, L., Kunert, K. J., Rennenberg, H., & Foyer, C. H. (1998). Glutathione: biosynthesis, metabolism and relationship to stress tolerance explored in transformed plants. *Journal of experimental botany*, *49*(321), 623-647.

Noctor, G., Gomez, L., Vanacker, H., & Foyer, C. H. (2002). Interactions between biosynthesis, compartmentation and transport in the control of glutathione homeostasis and signalling. *Journal of experimental botany, 53*, 1283-1304.

Oh, S. H., & Lim, S. C. (2006). A rapid and transient ROS generation by cadmium triggers apoptosis via caspase-dependent pathway in HepG2 cells and this is inhibited through N-acetylcysteine-mediated catalase upregulation. Toxicology and applied pharmacology, 212(3), 212-223.

Rubino, F. M. (2015). Toxicity of glutathione-binding metals: a review of targets and mechanisms. Toxics, 3(1), 20-62.

Singh, R., Gautam, N., Mishra, A., & Gupta, R. (2011). Heavy metals and living systems: An overview. *Indian J Pharmacol, 43*(3), 246-253. doi:10.4103/0253-7613.81505

Su, C. (2014). A review on heavy metal contamination in the soil worldwide: Situation, impact and remediation techniques. *Environmental Skeptics and Critics, 3*, 24.

Sumner, E. R., Shanmuganathan, A., Sideri, T. C., Willetts, S. A., Houghton, J. E., & Avery, S. V. (2005). Oxidative protein damage causes chromium toxicity in yeast. *Microbiology, 151*(Pt 6), 1939-1948. doi:10.1099/mic.0.27945-0

Suraweera, A., Münch, C., Hanssum, A., & Bertolotti, A. (2012). Failure of amino acid homeostasis causes cell death following proteasome inhibition. *Molecular cell, 48*(2), 242-253.

Thayyullathil, F., Chathoth, S., Hago, A., Patel, M., & Galadari, S. (2008). Rapid reactive oxygen species (ROS) generation induced by curcumin leads to caspase-dependent and -independent apoptosis in L929 cells. *Free Radic Biol Med, 45*(10), 1403-1412. doi:10.1016/j.freeradbiomed.2008.08.014

Vasireddi, M., Crum, A., May, H., Katz, D., & Hilliard, J. (2019). A novel antiviral inhibits Zika virus infection while increasing intracellular glutathione biosynthesis in distinct cell culture models. *Antiviral research, 161*, 46-52.

Verghese, J., Abrams, J., Wang, Y., & Morano, K. A. (2012). Biology of the heat shock response and protein chaperones: budding yeast (Saccharomyces cerevisiae) as a model system. *Microbiol. Mol. Biol. Rev.,, 76*, 115-158.

Vido, K., Spector, D., Lagniel, G., Lopez, S., Toledano, M. B., & Labarre, J. (2001). A proteome analysis of the cadmium response in Saccharomyces cerevisiae. *Journal of Biological Chemistry, 276*(11), 8469-8474.

Whitmarsh, A. J., & Davis, R. J. (1998). Structural organization of MAP-kinase signaling modules by scaffold proteins in yeast and mammals. *Trends Biochem Sci, 23*(12), 481-

Yokouchi, M., Hiramatsu, N., Hayakawa, K., Kasai, A., Takano, Y., Yao, J., & Kitamura, M. (2007). Atypical, bidirectional regulation of cadmium-induced apoptosis via distinct signaling of unfolded protein response. *Cell death and differentiation*, *14*(8), 1467.485. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/9868371

Zitka, O., Skalickova, S., Gumulec, J., Masarik, M., Adam, V., Hubalek, J., ... & Kizek, R. (2012). Redox status expressed as GSH: GSSG ratio as a marker for oxidative stress in paediatric tumour patients. *Oncology letters*, *4*(6), 1247-1253.

APPENDICES

Appendix A

List of genes in the Nanostring panel for *S. cerevisiae*

1. ADE5,7 - Bifunctional aminoimidazole ribotide synthase/glycinamide ribotide synthase
2. ADE6 - Phosphoribosylformylglycinamidine synthase
3. ADO1 - Adenosine kinase
4. CYS3 - cystathionine beta-synthase CYS3
5. CYS4 - cystathionine beta-synthase CYS4[
6. SAM1 - Methionine adenosyltransferase SAM1
7. SAM4 - S-adenosylmethionine-homocysteine S-methyltransferase SAM4
8. CUP1 - Cu Cd binding
9. GSH1 - Glutamate--cysteine ligase
10. GSH2 - Glutathione synthase
11. GLR1 - glutathione-disulfide reductase
12. MCA1 - Ca(2+)-dependent cysteine protease MCA1
13. OYE3 - NADPH dehydrogenase
14. PET9 - ADP/ATP carrier protein PET9
15. POR1 - Porin POR1
16. ATG1 - Serine/Threonine protein kinase ATG1
17. ATG2 - Atg2p
18. ATG4 - Atg4p
19. ATG6 - Beclin 1
20. ATG7 - Atg7p

21. ATG8 - Atg8p
22. SEC4 - Rab family GTPase SEC4
23. COX2 - Cytochrome c oxidase subunit 2
24. CDC19 - Pyruvate kinase CDC19
25. ENO1 - Phosphopyruvate hydratase ENO1
26. FBA1 - Fructose-bisphosphate aldolase FBA1
27. PDB1 - Pyruvate dehydrogenase (acetyl-transferring) subunit E1 beta
28. PFK1 - 6-phosphofructokinase subunit alpha
29. PGI1 - Glucose-6-phosphate isomerase
30. PGK1 - Phosphoglycerate kinase
31. TDH1 - Glyceraldehyde-3-phosphate dehydrogenase (phosphorylating) TDH1
32. TDH2 - Glyceraldehyde-3-phosphate dehydrogenase (phosphorylating) TDH2
33. TDH3 - Glyceraldehyde-3-phosphate dehydrogenase (phosphorylating) TDH3
34. TPI1 - Triose-phosphate isomerase TPI1
35. ALG9 - dolichyl-P-Man:Man(6)GlcNAc(2)-PP-dolichol alpha-1,2-mannosyltransferase
36. TAF10 - Taf10p
37. TFC1 - transcription factor TFIIIC subunit TFC1
38. UBI4 - ubiquitin
39. KAR2 - Hsp70 family ATPase KAR2
40. PDI1 - protein disulfide isomerase PDI1
41. SSC1 - Hsp70 family ATPase SSC1
42. SSE1 - Adenyl-nucleotide exchange factor SSE1
43. IPP1 - Inorganic diphosphatase IPP1

44. KTR7 - putative mannosyltransferase
45. YPT32 - Rab family GTPase YPT32
46. FKH1 - Forkhead family transcription factor FKH1
47. FKH2 - Forkhead family transcription factor FKH2
48. GAD1 - Glutamate decarboxylase GAD1
49. GRX2 - Dithiol glutaredoxin GRX2
50. GRX5 -
51. HCM1 - monothiol glutaredoxin GRX5
52. HSP30 - Hsp30p
53. MCR1 - cytochrome-b5 reductase
54. MSN2 - stress-responsive transcriptional activator MSN2
55. MSN4 - stress-responsive transcriptional activator MSN4
56. MXR1 - peptide-methionine-S-sulfoxide reductase
57. SIR2 - NAD-dependent histone deacetylase SIR2
58. SSA1 - Hsp70 family ATPase SSA1
59. SSA4 - Hsp70 family chaperone SSA4
60. SSB1 - Hsp70 family ATPase SSB1
61. SSB2 - Hsp70 family ATPase SSB2
62. AHP1 - thioredoxin peroxidase AHP1
63. GPP1 - glycerol-1-phosphatase RHR2
64. HSP31 - glutathione-independent methylglyoxalase
65. TSA1 - thioredoxin peroxidase TSA1
66. HSP26 - Chaperone protein HSP26

67. DDR48 - DNA damage-responsive protein 48
68. RRN5 - Rrn5p
69. BUD21 - Bud21p
70. EFB1 - Translation elongation factor 1 subunit beta
71. HYP2 - Translation elongation factor eIF-5A
72. RPP0 - Ribosomal protein P0
73. EFT2 - Elongation factor 2
74. VMA2 - H(+)-transporting V1 sector ATPase subunit B
75. SEC53 - phosphomannomutase SEC53

Appendix B

A survey of intraocular expression of immune response genes during onset and progression of experimental murine cytomegalovirus (MCMV) retinitis in mice and retrovirus-induced immunosuppression (MAIDS).

In this series of experiments, MAIDS infected mice were treated with MCMV virus by direct subretinal injection into the left eye and a carrier was subretinally injected into the right eye as a control. Eyes were collected on days three, six, and nine for expression assay with a Nanostring panel (nCounter® Immunology Panel (Mouse)). The above-described series of experiments was designed by PhD candidate Jessica Carter under the direction of Dr. Richard Dix at GSU, and I provided support by running the Nanostring panel and aided in the analysis of the data. Nanostring data were used to validate and verify qPCR expression analysis undertaken in the previous series of the same experiment. The aid provided by this researcher to the Dix lab was enough to grant tertiary authorship on a paper with the same title pending publication.