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STUDY OF CELLULAR ACTIVITIES IN RESPONSE TO METAL-INDUCED APOPTOSIS
IN *SACCHAROMYCES CEREVISIAE* USING FTIR

by

RUPA KODURU

Under the direction of Dr. John E. Houghton

ABSTRACT

Saccharomyces cerevisiae exhibits an apoptotic response upon exposure to toxic metals such as cadmium (Cd) and copper (Cu). Preliminary findings indicate that this response is dependent –to some extent- on the presence of a fermentable carbon source, glucose. To investigate this dependency we monitored the apoptotic response to both metals in the presence and absence of glucose and have shown that glucose is absolutely necessary in order to induce apoptosis in yeast at least during the exposure to metal. We have also looked at the biochemical changes that are taking place in yeast when treated with Cd using Fourier Transform Infra-Red (FTIR) Spectroscopy. Our results suggest that there are definitive changes in cellular activities that are discernable at $1660\text{-}1640\text{cm}^{-1}$ (amide I), $1540\text{-}1510\text{cm}^{-1}$ (amide II) and $1140\text{-}1080\text{cm}^{-1}$ (DNA absorption bands).

INDEX WORDS: Heavy metals, Oxidative stress, Yeast, Apoptosis, FTIR

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RUPA KODURU

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

2010

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2010

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INTRODUCTION:

Heavy metals and their toxicity:

Heavy metals are essential for living organisms, but when present in relative excess they have toxic and harmful affect on biological systems. In all living organisms, heavy metals are needed for maintaining protein structure or for catalytic activity (Waldron, Rutherford et al. 2009). So it is important to maintain optimal levels of metal intake to reduce their damaging effects. When these metals accumulate in the cell, or body of higher organisms, they can often act as carcinogens, with their toxic effects in higher organisms ranging from a series of degenerative disorders to chronic genotoxic effects resulting in protein damage or lipid peroxidation (Nargund, Avery et al. 2008). Such deleterious consequences of excess heavy metal exposure or build up, and their influence upon a number of diseased states, has spurred the research leading to a more thorough understanding of the roles metals play in the normal maintenance of cellular functions, as well as the response of both prokaryotic and eukaryotic organisms to acute and chronic exposure to the different heavy metals (Wysocki and Tamas 2010). Even so, little is known about how heavy metals act at the molecular level in higher eukaryotes. To offset this deficit researchers have used *Saccharomyces cerevisiae* as a model organism to gain some insights into the molecular details as to exactly how cells respond to heavy metal exposure, and the cascade of events by which they are able to offset some of the more damaging aspects of metal –induced cellular toxicity(Wysocki and Tamas 2010). Principal among these, exposure of cells to relatively low levels of heavy metals has been shown to result in a metal-induced oxidative stress that results in a variety of cellular damage ranging from: impaired DNA repair or by inhibiting the function of proteins that are involved in cell cycle progression, apoptosis, cell proliferation or differentiation (Fig 1).

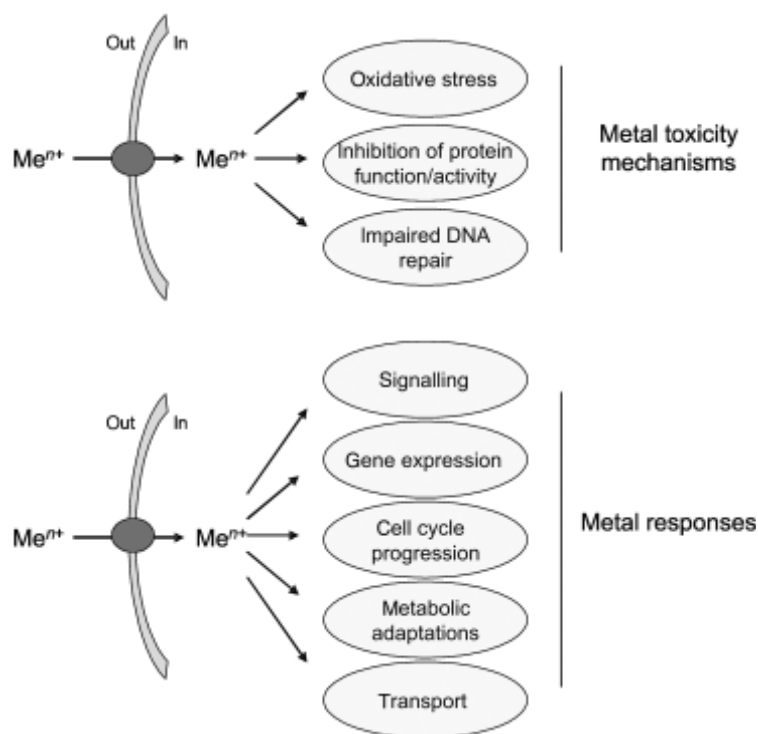


Figure 1: Metal responses and their toxicity mechanisms

Metal become toxic directly or indirectly, by triggering oxidative stress in cells or by interfering with some proteins function and/or by impairing DNA repair mechanisms. But in response to metal exposure other events like altering gene expression and metabolic pathways, and others as shown(Wysocki and Tamas 2010).

As a consequence of these known pathways of cellular damage and toxicity, heavy metals have often been divided into two groups, *redox-active* (Fe, Cu...) and *redox-inactive* (Cd, Cr, Pd, Hg...) metals, as a function of their inherent ability to generate reactive oxygen species (ROS) directly or indirectly(Nargund 2010; Wysocki and Tamas 2010).

Research undertaken in this work has focused mainly upon some of the more immediate cellular responses that underpin the oxidative stress in *S. cerevisiae* brought about by exposure to two such heavy metals, cadmium and copper, as representative metals for each type of redox-activity within the cell(Nargund 2010).

Oxidative stress due to metals:

In recent years, several studies have elucidated the production of ROS and their specific role in causing oxidative stress in different organisms (Costa and Moradas-Ferreira 2001). So how are these ROS produced? Reactive Oxygen Species (ROS) are formed as by-products of cellular metabolism and enzymatic activities within organelles such as the mitochondria. ROS produced from these biochemical events occurring in mitochondrial respiration constitute almost the entire metabolic consumption of oxygen in the cell (Gutteridge and Halliwell 1999). The superoxide, hydroxyl radicals and hydrogen peroxide radicals, collectively called ROS, cause oxidative damage to DNA and proteins (Gutteridge and Halliwell 1999). Under normal conditions, the damaging effects of ROS are neutralized by enzymes such as glutathione (GSH) peroxidases, superoxide dismutase (SOD), etc., thereby preventing further cellular damage due to excessive production of ROS generated from Haber-Weiss or Fenton's reactions. But under certain aberrant conditions that lead to stress, the amount of ROS produced will exceed the handling capacity of the cell's antioxidant mechanisms, resulting in oxidative stress. In higher organisms, such metal-induced oxidative stress can result in the development of several neurological disorders such as: Alzheimer's disease, Parkinson's disease and familial amyotrophic lateral sclerosis (Costa and Moradas-Ferreira 2001; Nargund 2010).

Copper (Cu) is a heavy metal that serves as protein cofactor in a number of essential biological activities, such as elimination of free radicals, respiration and various neurological functions (Kuo, Zhou et al. 2001). Copper is known to be a *redox-active* heavy metal in that it can generate ROS (hydroxyl radicals and oxygen radicals) directly through the Haber-Weiss and Fenton reactions (Aydin, Celik et al. 2003; Haynes, Titus et al. 2004; Nargund 2010). The excess ROS that are generated can cause considerable damage to all the major cellular components

such as proteins, lipids and DNA leading to marked oxidative stress(Wysocki and Tamas 2010). Thus, any imbalance in the normal copper homeostasis within the cell can result in genetic disorders such as Menke's and Wilson's disease. Menke's syndrome is an X-chromosome linked and caused due to improper mutations in copper transporting genes (Andrews 2001). Such mutations can give rise to a number of diseased states, such as Wilson's disease, in an autosomal recessive linked trait that results in the accumulation of copper in kidneys, liver and intestinal mucosa, and leading to its insufficiency in other body tissues (Valko, Morris et al. 2005). The role of copper in other diseased states, such as Alzheimer's disease, has also been studied, and found to be responsible for the directed damage of a protein known as LRP, which is involved in transporting amyloid-beta out of the brain into the body(Valko, Morris et al. 2005; UPMC 2007). Indeed, copper-induced oxidative stress has been implicated in a number of neurological abnormalities (Valko, Morris et al. 2005).

Unlike Cu, Cadmium (Cd) is a *redox-inactive* metal and is one of the most toxic heavy metal that results in oxidative stress when present in excess. Cd generates ROS indirectly by replacing metals like iron and copper from enzymes and intracellular depots such as ferritin and apoferritin, -indirect sources for oxidative stress (Price and Joshi 1983). It also inhibits the expression of anti-oxidant enzymes such as SOD and catalase, indirectly leading to increases in ROS (Fig 2). Several carcinogenicity studies using Cd have revealed that it can induce expression of stress response genes, such as those encoding metallothionin (MT), Heat Shock Proteins (HSPs) and Glutathione (GSH) synthesis and several other genes (Casalino, Calzaretto et al. 2002; Henkler F. 2010).

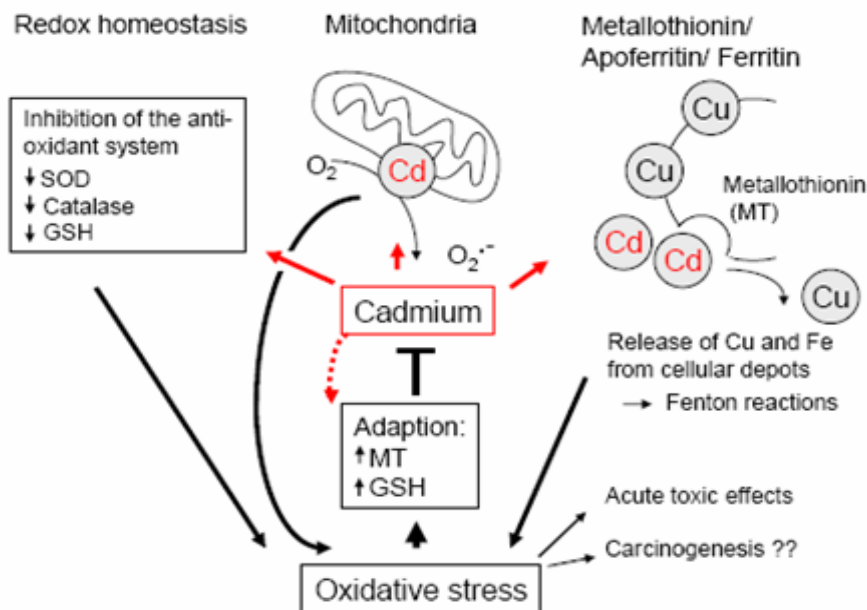


Figure 2: Cadmium induced Oxidative stress mechanisms

Cadmium belongs to a *redox-inactive* metal group. This figure shows different mechanisms that lead to the production of ROS. Cadmium induces expression of metallothionin (MT) and glutathione (GSH) in response to oxidative stress, thus leading to the inhibition of ROS production (Henkler F. 2010).

Yeast as a Model Organism:

When heavy metals are taken up by the cells it is accepted that the primary mechanism of cellular damage is generated by the excessive production of ROS, causing oxidative stress and related cellular damage (Fig 3). Yeast can serve as an excellent model system for studying the effects of such metal toxicity since its genome and proteome are well characterized (Madeo, Frohlich et al. 1999). Being a single celled organism it makes it easy to manipulate when compared to complex higher eukaryotes (Nargund 2010).

Just prior to the turn of the last century Madeo and his group showed that several pro- and anti-apoptotic genes from mammals could be expressed in yeast and give rise to an apoptotic response -suggesting that there is a strong evidence of a common origin of the apoptotic process

in yeast(Madeo, Frohlich et al. 1999). Thereafter, orthologs of genes such as caspase, *AIF-1*, *BIR-1* were expressed during yeast apoptosis (Madeo, Frohlich et al. 1999). Subsequently it has been demonstrated that yeast undergoes apoptosis in response to oxidative stress, as seen in higher eukaryotes(Costa and Moradas-Ferreira 2001).

Yeast cells share common features both at organelle and macromolecular level with mammalian cells. Some yeast proteins have been characterized which are highly homologous with human proteins(Costa and Moradas-Ferreira 2001). Another advantage of using yeast is, it can be grown on different fermentative and non-fermentative carbon sources to control the mode of respiration, thus involving mitochondria in different cellular activities(Nargund 2010). Thus, yeast is considered one of the best model systems to elucidate molecular mechanisms leading to oxidative stress responsible for problems related to apoptosis, aging and neurodegenerative disorders such as Alzheimer's and Parkinson's disease.

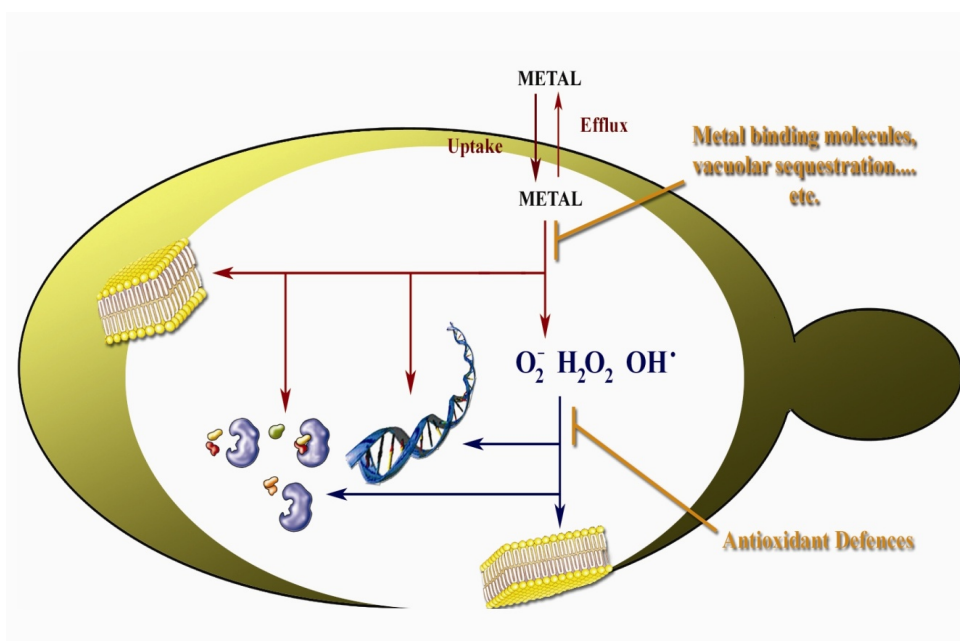


Figure 3: ROS generation and Oxidative stress in budding yeast

Metals when taken up by the cells, generate ROS and cause oxidative stress by damaging cellular macromolecules(Houghton 2010).

Apoptosis due to metals:

The above mentioned metals not only cause metal toxicity but also results in apoptosis due to oxidative stress in several organisms. Apoptosis is referred to as Programmed Cell Death(PCD), in which cells commit suicide(Liu and Mantsch 2001). This cellular “suicidal” mechanism is of primary importance in development and in maintaining homoeostasis of multi-cellular organisms (Madeo, Frohlich et al. 1999; Liang and Zhou 2007). In many organisms, uncontrolled apoptosis leads to cancer, aging, neurological disorders and tumor suppression(Liang and Zhou 2007). It is during this process, cells undergo series of events such as condensed chromatin, DNA fragmentation, mitochondria perturbation, shrinkage of cells and finally, the cells are broken down into small apoptotic bodies and are phagocytosed simultaneously (Fig 5) (Madeo, Frohlich et al. 1999). Thus apoptosis is an active process requiring more energy and involving complex signaling events for controlling it. In humans, apoptosis occurs by two different pathways such as extrinsic and intrinsic pathways. In extrinsic pathway, caspases get activated when ligands are binded to receptors and activated caspases fragment cell’s DNA leading to apoptosis. Whereas in intrinsic pathway, cells receive internal signals derived either from excessive ROS production that leads to oxidative damage or from mutations in mitochondrial DNA. It is indicated that reactive oxygen species (ROS) plays an important role for intrinsic pathway of metal-induced apoptosis from various studies (Jamers, Van der Ven et al. 2006; Nargund 2010).

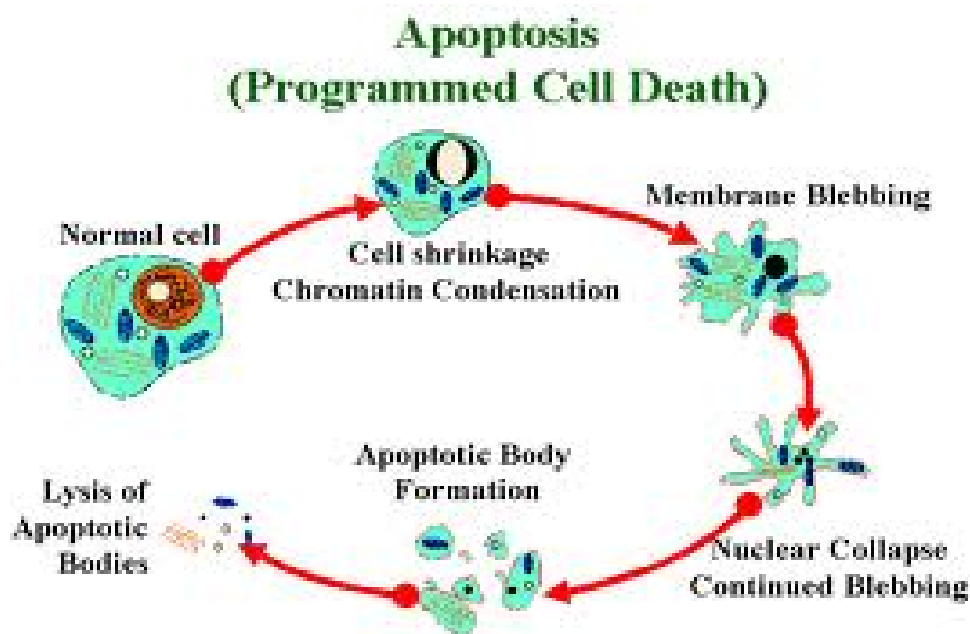


Figure 4: Apoptosis- Programmed Cell death

In this figure, cells undergoing apoptosis show certain features such as DNA damage, chromatin condensation, cell shrinkage, nuclear collapse and membrane blebbing(2010).

Similar to multi-cellular organisms, unicellular organisms, like *S. cerevisiae*, also undergo apoptosis due to oxidative stress, providing a way for exploring the important apoptotic regulators in different organisms and by serving as a key model system(Madeo, Frohlich et al. 1999; Nargund, Avery et al. 2008). In *S. cerevisiae*, apoptosis has been shown to take place when cells are exposed to toxic metals like cadmium, chromium, copper, manganese, lead and other various metals. It is that in yeast, only mitochondria-mediated apoptosis thought to occur by causing oxidative stress. It is also that the apoptotic events are conserved in yeast by causing mitochondrial perturbation and release of cytochrome c, caspase activation, DNA damage, chromatin condensation, etc., (Liang and Zhou 2007; Nargund, Avery et al. 2008).

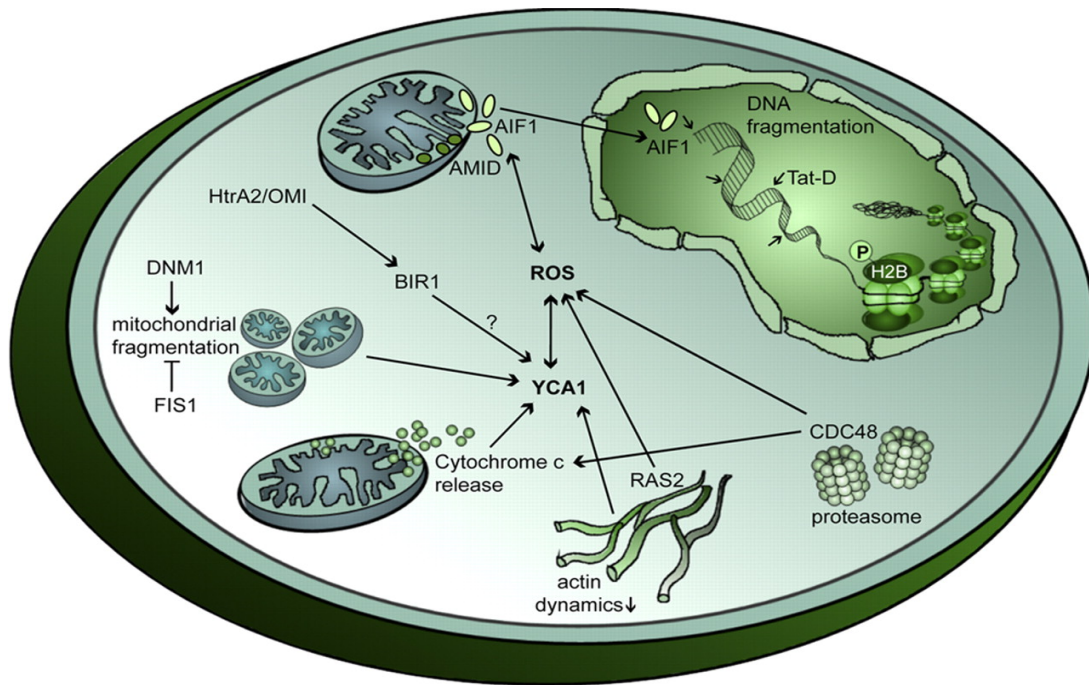


Figure 5: Conservation of Apoptotic events in *S. cerevisiae*

The series of events like cytoskeletal perturbation, increase of Reactive Oxygen Species (ROS) in the cytosol, dispersion of proteins involved in the activation of yeast caspase (Yca1p), mitochondrial fragmentation, DNA damage and chromatin condensation in the nucleus, happening during apoptotic response along with cytochrome c, process similar to eukaryotic apoptosis (Buttner, Eisenberg et al. 2006).

FTIR PRINCIPLE AND ITS APPLICATIONS:

In recent years, the extensive application of Fourier Transform Infra-red (FTIR) Spectroscopy in medicine and biology has been very useful for researchers to obtain information on all macromolecules inside a cell population(Liu and Mantsch 2001). It emerged as a critical tool for elucidating the biochemical mechanisms that occur in cells due to various external factors(Silvia Gaudenzi 2004). The principle of FTIR spectroscopy is based on Michelson interferometer principle, where the infrared light is passed through the sample, and in which some amount of light is absorbed by the sample while reflecting the unique vibrations created by similar molecules forming a molecular fingerprint in the form of a spectrum for that sample. The interferogram generated is converted into a spectrum by applying Fourier transform's equation (Fig 6). Thus, the IR spectra obtained provides both quantitative and qualitative information about the molecules.

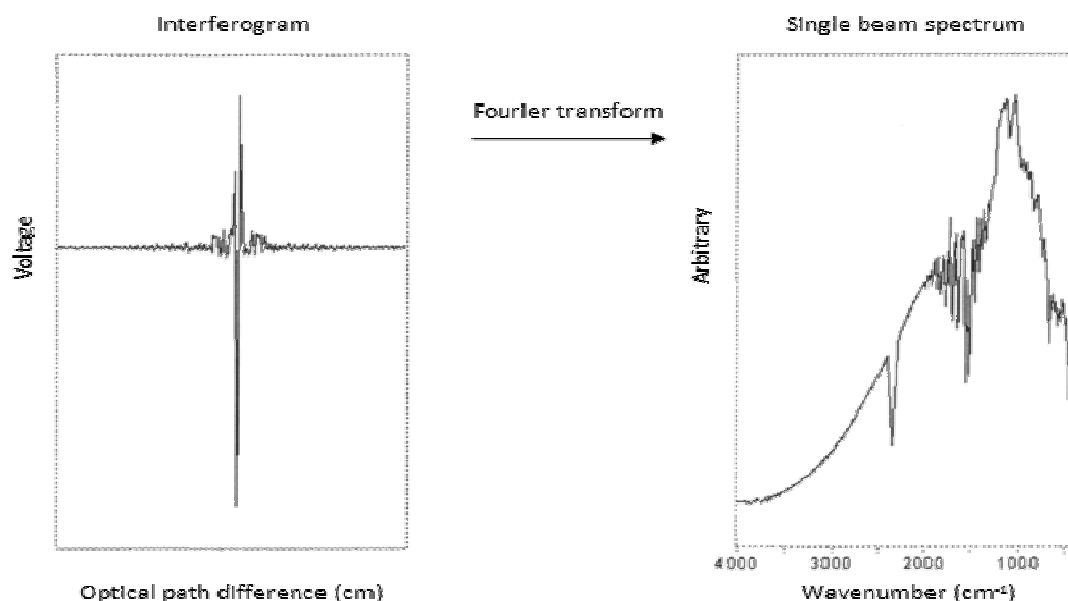


Figure 6: Conversion of interferogram into spectrum

Fourier transforms equation is applied in converting an interferogram, which is a time domain signal to the frequency domain spectrum(Smith 1996).

The quantitative data is obtained from the intensities of the spectra and the qualitative data like the environment, structure and bonding information of a molecule is obtained from absorption spectra (Liu, Shi et al. 2005). The absorption bands produced from the bonds found in macromolecules are visible in Fig 7. As shown in the figure the region from 4000 to 3100cm^{-1} shows the bands that result from O-H and N-H stretching and the region from 3100 to 2800cm^{-1} display the stretch of methyl groups from lipids. The bands from 1800 - 800cm^{-1} represents amide I bonds and bands from 1650 - 1540cm^{-1} gives amide II bonds vibrations. The bands ranging from 1100 - 750cm^{-1} represent the stretching pattern of phosphate bonds in nucleic acids and other carbohydrate structures. Thus, the contribution from all these data makes up the complete IR spectrum.

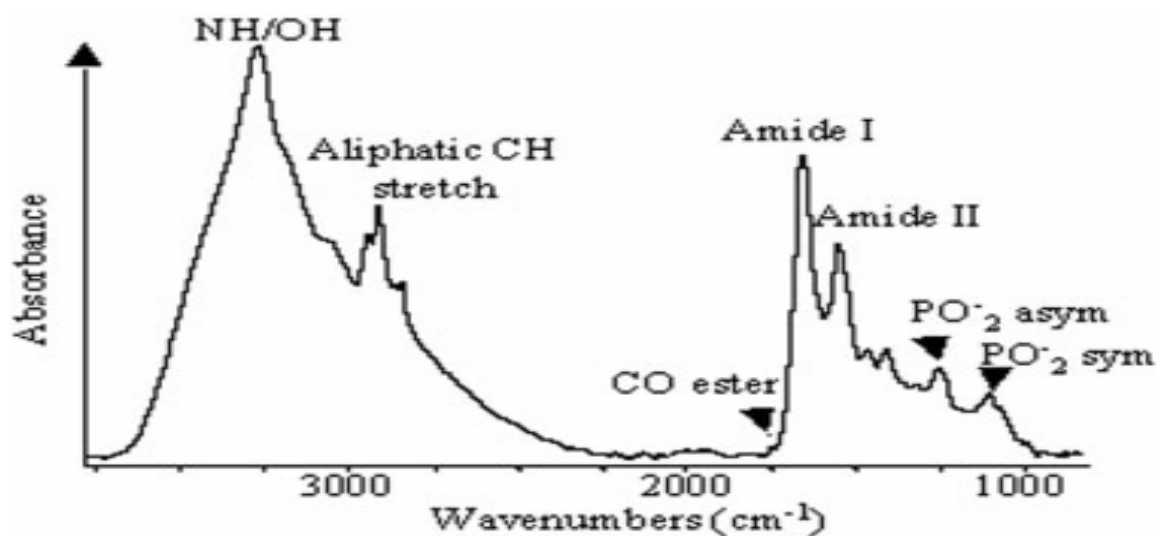


Figure 6: Typical spectrum produced for Jurkat T-cells

The above figure shows the spectra of Jurkat T cell nucleus obtained from an FTIR measurement (Jamin, Miller et al. 2003).

Experimental Question:

As discussed, heavy metals like Copper (Cu) and Cadmium (Cd) are shown to be involved in a variety of neurodegenerative disorders. Cadmium (as a redox-inactive metal) and copper (as a redox-active metal) both produce ROS -causing oxidative stress in the cell. ROS produced by these metals are known to cause damage to macromolecules like proteins, lipids and nucleic acids resulting in apoptosis. In *S. cerevisiae*, these metals are thought to induce apoptosis by increasing ROS production, mitochondrial membrane hyperpolarization and activating yeast caspase (Yca1p). Thus, yeast can be used as a good, simplified model for studying the effects of ROS on cellular structures and perhaps, the mechanisms that underlie heavy metal toxicity in the cell.

Amrita Nargund, in this laboratory, has previously been able to show that low concentrations (30 μ M cadmium and 8mM copper) have an acute toxic effect upon yeast, in that within one hour of exposure, a sub population of the exposed cells initiate a caspase-specific programmed cell death (Nargund, 2008; Nargund, 2010). She further showed that such an acute cellular response to metal exposure was apparent even if the metal were removed from the growth medium after only 1hour; suggesting that even 1hour exposure is sufficient to elicit the caspase-specific apoptotic response of the cells to such low concentration of heavy metal (Nargund, 2008; Nargund, 2010).

When analyzing the response of cells to cadmium or copper, up to 10-20% of cells exhibited the caspase activity and ultimately underwent programmed cell death. If glucose was removed from the growth media, however, all apoptotic response was lost, indicating that glucose, as well as caspase (Yca1p), is also necessary for yeast cells to undergo apoptosis.

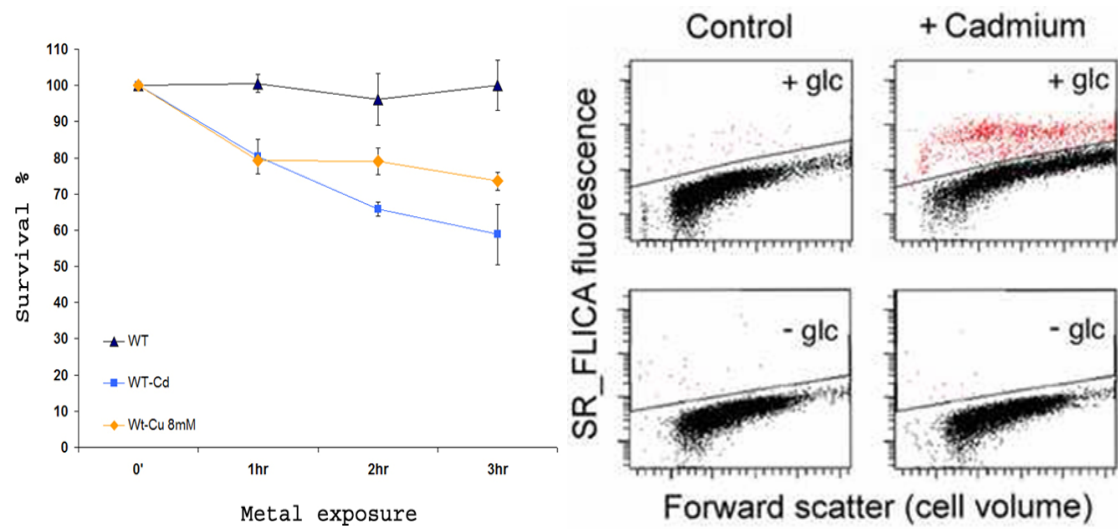


Figure 8: Cell death and caspase activity when cells treated with metals

The upper panels showing the caspase activity when cells treated with cadmium and having glucose during recovery period. The lower panels shows that there is no caspase activity when glucose is not present during the recovery period after exposing the cell to Cadmium (Nargund 2010).

Hypothesis 1: Glucose is absolutely necessary for yeast cells to undergo apoptosis when treated with heavy metals, cadmium and copper.

Having observed that yeast cells exhibit an apoptotic response even when exposed to heavy metals, cadmium and copper, for as little as 1 hour, and allowed to recover for up to 3 hours thereafter. We have tried to demonstrate the absolute requirement of glucose throughout this response phase of the cells –from the initial 1h period of metal exposure, to the subsequent recovery period (3h) in which no metal is necessary. Using both cadmium and copper as representative heavy metals, and YCA1 mutant cells as controls, we have undertaken a series of experiments to confirm the importance of glucose in the caspase-dependent apoptotic response of cells, as well as to demonstrate whether the requirement for glucose is metal specific.

Hypothesis 2: FTIR can be used for looking at metabolic changes taking place in yeast during metal-induced apoptosis

We used Fourier Infra-Red Spectroscopy (FTIR) to observe the metabolic changes in yeast following metal exposure over a period of time. In this way, we tried to demonstrate that yeast cells undergo significant metabolic changes in response to the presence of metals. Furthermore, considering the presence of heavy metals, we want to determine whether these changes are a generalized cellular response of yeast or whether these metabolic changes are specific to the sub-population of yeast that undergo apoptosis. To verify that, we performed cell sorting techniques to address cellular responses in different apoptotic and non-apoptotic cells, and then analyze changes in metabolic activity using FTIR.

MATERIALS & METHODS:

Culture and treatments:

Saccharomyces cerevisiae BY4741 parental Wild Type and mutant strain, YCA1 were cultured in YEP broth [2 % (w/v) peptone (BD), 1 % (w/v) yeast extract] with either additional 2 % (w/v) dextrose or 3% (w/v) glycerol. The cells were incubated overnight at 30°C and harvested at OD₆₀₀ 2.0.

At OD₆₀₀ 2.0 cells were exposed to heavy metals Cadmium nitrate, Cd (NO₃)₂ [30 µM] and Cu (NO₃)₂ [8mM] for 1h in the presence of glucose(**gluc**) or glycerol (**glyc**) containing medium. Cell were harvested by centrifugation and the pellet was resuspended in YEP containing glucose (**gluc**) or glycerol (**glyc**) or nothing and diluted to OD₆₀₀~1.0 and post-incubate for 3h.

Flow cytometry:

Intracellular ROS that was produced in cadmium treated cells were detected using the oxidant – sensitive dye dihydro-rhodamine123 (DHR123- Sigma Aldrich). DHR123 was added at 5 µg per ml of cell culture, from a 2.5 mg⁻¹ stock solution in ethanol. Cells were then incubated at 30°C for 3 hr and fluorescence was determined using flow cytometer FACSCanto (BD Biosciences) at 525nm. To determine mitochondrial membrane potential, we used Rhodamine-123 with a final concentration of 2µM and then cells were incubated for 10min before we analyzed with the cytometer at an emission wavelength of 535nm.

For copper treated cells, intracellular ROS were detected using the oxidant- sensitive dye Dihydroethidium (DHE, Sigma Aldrich). Cells were then quantified on FACSCanto at 590nm emission filter.

Sample preparation for FTIR measurements:

Cells were grown until OD₆₀₀ 2.0 as mentioned above. Samples of cell suspensions were taken after cells reached OD₆₀₀ 2.0 (time 0). Cells were harvested by centrifuging at 14,000 rpm for 2 min at 4°C and then washed three times with 1 ml of sterile distilled water for removing cadmium residue that is left over. The washed cells were then suspended in sterile water until all the samples reach OD₆₀₀ 2.0-3.0. 0.5 µl of each suspension was spread on Low-e microscope slide that supports for FTIR measurements respectively. The sample drops were air-dried before taking to measurements.

Both WT (BY4741) and YCA1 cells that were prepared as mentioned above were taken in the following order: Untreated cells that don't undergo any chemical treatment were considered as control and the samples were spreaded on the slide at 60min, 120min, and 180 min time intervals. Cells treated with 30 µM of Cd (NO₃)₂ were considered as treated (test) and the samples were taken in the similar manner as before.

FTIR measurements:

Mid-infrared spectra were acquired in the 4000-200 cm⁻¹ range by using a Varian 7000 spectrometer coupled to a Vis/IR microscope equipped with a photoconductive MCT detector and a 10X objective for reflection measurements. For each sample, about 8-10 point by point spectra were acquired at 2 cm⁻¹ resolution on a 35 µm-40 µm area by co-adding 64 scans in reflection.

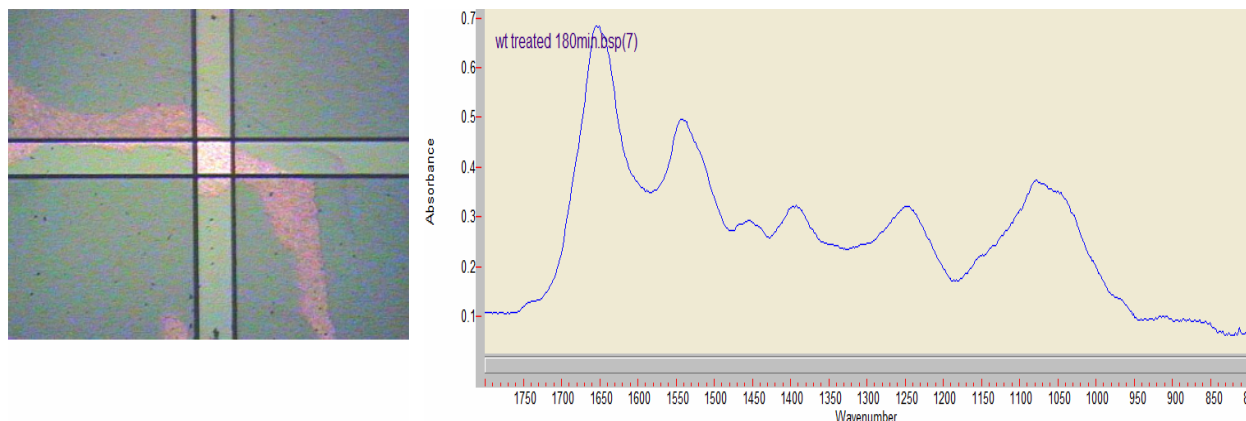


Figure 9: Sample spread on Low-e microscopic slide and typical spectrum produced by FTIR in the area of $40 \times 40 \mu\text{m}^2$ as shown

Cell Sorting:

Cells were grown overnight until OD 2.0 as mentioned above and treated with $30 \mu\text{M}$ $\text{Cd}(\text{NO}_3)_2$ for 1 hr. After 1h, cells were washed and harvested in fresh YEPD medium and incubated for 3 hours at 30°C . Intracellular ROS were detected using the oxidant- sensitive dye Dihydroethidium (DHE, Sigma Aldrich). Cells were then sorted on FACS Aria Cell Sorter (BD Biosciences) at 590nm emission filter with an average of 23,000 – 25,000 cells per sec.

The cells exhibiting ROS were taken as apoptotic population and the rest were treated as normal cells. Then the collected cells from sorting were centrifuged and washed and resuspended in sterile water and taken for the FTIR measurements as mentioned above.

RESULTS & DISCUSSION:

Nargund and others in the laboratory have previously demonstrated that even relatively low concentrations of either cadmium (30 μ M) or copper (8mM) were able to induce an immediate oxidative-stress in yeast, and that this stress results in the rapid onset (within 4h) of programmed cell death in a subset of the population of exposed cells (Nargund, 2008, 2010). Both Nargund and Shanmuganathan had previously shown that the immediate cellular responses to both copper and cadmium (such as directed oxidation of proteins) peaked within 1h of exposure (Shanmuganathan, 2004; 2007), and that this limited exposure to cadmium or copper was sufficient to elicit a subsequent, apoptotic response in some of the cells, which accounted for any and all loss of cell viability as a result of exposure to metals (Nargund, 2008; 2010). The cellular responses that were monitored throughout these series of experiments included mitochondria perturbation and increased ROS presence within a sub population of stressed cells, along with an assessment of caspase activation and cellular damage, such as mitochondrial dysfunction and protein oxidation brought about by metal-induced oxidative stress within the cells (Shanmuganathan, 2004 and Nargund 2008).

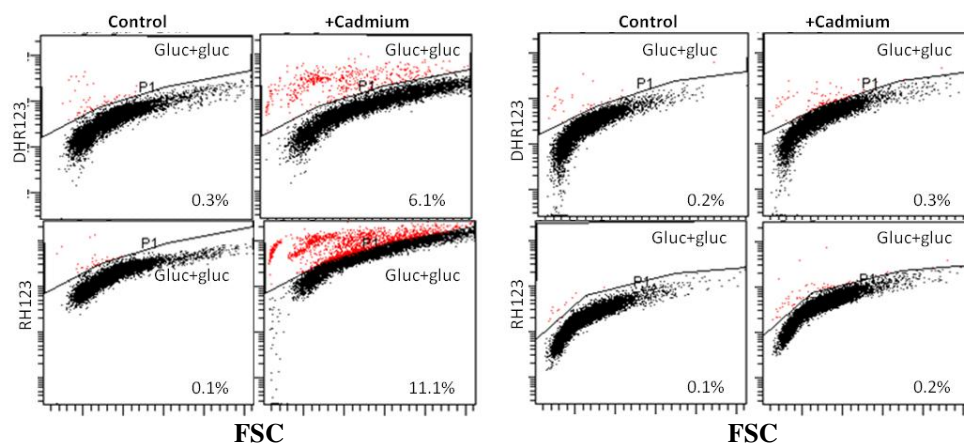
All these analyses, however, were carried out on cells growing in rich media and in the presence of a fermentable carbon source, glucose. Moreover, the initial findings by Shanmuganathan indicated that mutants in the glycolytic pathway abrogated the apoptotic response to metal-induced oxidative stress (Shanmuganathan, 2007 dissertation). Consequently, the importance of glucose to the apoptotic process needed to be ascertained.

In an effort to begin to determine how important glucose is to the process, the response of yeast to heavy metals was analyzed with respect to the presence or absence of glucose in the growth medium. Of the various cellular responses that have previously been used to characterize the apoptotic response of yeast cells to the presence of heavy metals, elevated cellular ROS levels and mitochondrial dysfunction have provided some of the more definitive, early effects that preempt cellular dysfunction that ultimately leads to programmed cell death. Both these cellular response markers were used to try to determine the role that glucose plays in the apoptotic response of yeast to heavy metals. Furthermore, in order to analyze the role of carbon source in the apoptotic process, the effect of metal exposure on Wild Type (WT) and $\Delta YCA1$ (a yeast caspase deletion mutant that is unable to undergo caspase-specific apoptosis) was determined under a variety of growth conditions.

As mentioned previously, it has already been shown that a minimal exposure time for metals to exert a discernable apoptotic effect (1 h; Shanmuganathan, 2004; Nargund, 2008) is necessary, followed by a “refractory period” (3 – 4 hr post exposure), in which time a subset of cells within the population begin to exhibit discernable signs of oxidative stress (Nargund, 2008). In the first series of experiments, therefore, cells were exposed to the redox-inactive cadmium ($30\mu\text{M Cd (NO}_3)_2$) for 1h in the presence or absence of glucose, and these cells were then allowed to recover for 3h in fresh YEPD growth medium, as detailed previously (Nargund, 2008). Variations in ROS levels were observed using the fluorescent dye, DHR123, which has

the ability to permeate through cell walls and fluoresces in a quantitative manner when oxidized into a fluorescent product. Similarly, mitochondrial dysfunction was also quantitatively measured by monitoring the uptake of the cationic, lipophilic dye Rhodamine 123 (RH123; Nargund, 2008). The results depicted in Fig. 10 show that, with WT cells, 10-15% of the exposed cells exhibit excessive amounts of ROS, confirming that, within four hours of initial exposure to cadmium, caspase activation is required before any significant ROS production is apparent as there was no apoptotic response in caspase deficient mutant $\Delta YCA1$. Moreover, these results also demonstrate the critical requirement for glucose to be present in the medium during the “refractory period”, if any ROS is to be produced, suggesting that glucose is also important for any apoptotic response to occur within this time. The importance of glucose was further evidenced by analyzing mitochondrial membrane hyperpolarization in cells treated in as similar way to that in Fig.10.

(A) WT cells (BY4741)

(B) $\Delta YCA1$ cells

(C) WT cells (BY4741)

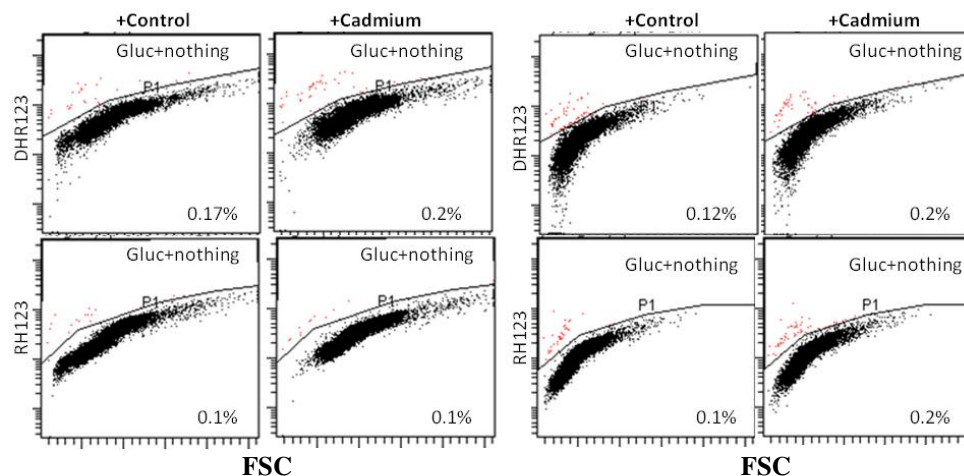
(D) $\Delta YCA1$ cells

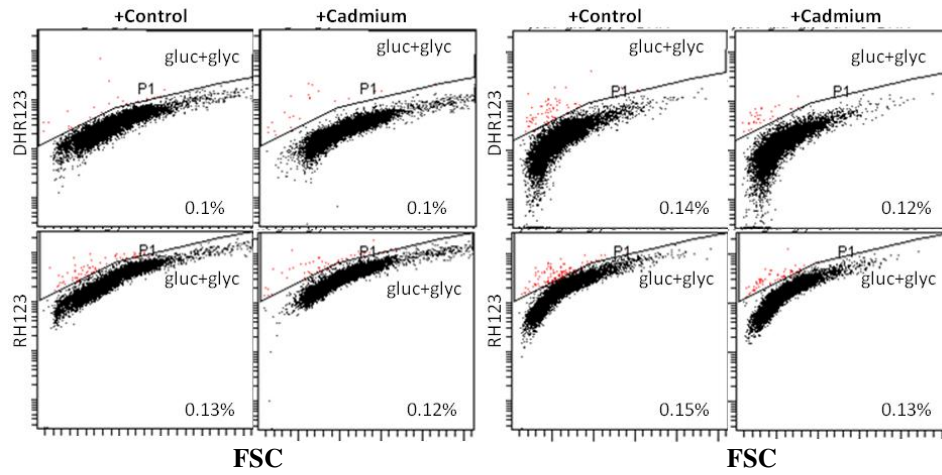
Figure 10: Cadmium induces mitochondrial membrane hyperpolarization and enhanced ROS levels in presence of glucose and yeast caspase (Yca1p)

Cells were induced with $30\mu\text{M Cd (NO}_3)_2$ for 1h and then recovered 3h in fresh medium with no cadmium. Intracellular ROS were detected using the oxidant-sensitive fluorescent dye DHR123. Cells that were induced with Cd were stained with DHR123 during the post-incubation period (3hrs) and the fluorescence was analyzed with flow cytometer. Control cells were treated in exactly the same way as the +Cd treated cells except the absence of Cd. Changes in the mitochondrial membrane potential as a consequence of +Cd treatment was determined using RH123. The treated and the control cells were stained before they were taken to analyze under flow cytometer. (A) & (B) WT cells were able to show increase in ROS and membrane hyperpolarization in the presence of glucose and caspase, Yca1p whereas YCA1 mutant do not (C)&(D) Neither WT or YCA1 mutant cells did not show any apoptotic response when glucose is removed during refractory period *Note: (A) & (B) Gluc+Gluc indicate the presence of glucose during 1h of metal exposure and also during 3h recovery period (C) & (D) Gluc+nothing indicate the presence of glucose during 1h of metal exposure and no carbon source provided during 3h recovery period

To investigate further the role of glucose during metal induced apoptosis in yeast the glucose was replaced by a non-fermentable carbon source, glycerol. In order to serve as a carbon source, glycerol is converted to glycerol-3-phosphate by glycerol kinase, and then transformed into DHAP by glycerol-3-phosphate dehydrogenase which acts as a substrate in gluconeogenesis. Thus glycerol can be used as a sole carbon source under aerobic conditions in *S. cerevisiae*.

Experiments were carried out in a similar manner to those shown previously, with the exception that the cells were exposed to cadmium for 1h in the presence of glucose, but were then centrifuged and resuspended in media containing 3% glycerol for 3h and analyzed, as detailed previously. None of these cells exhibited any signs of enhanced production of ROS, and neither WT or $\Delta YCA1$ mutant cells showed any signs of an apoptotic response to the presence of cadmium (Fig. 11 A & B). Curiously, in cells that were exposed to cadmium in the presence of glycerol only (Fig. 11 C & D) a small population of both WT and mutant $\Delta YCA1$ demonstrated some heightened levels of ROS. The finding that both cell types gave rise to similar populations of cells with significant levels of ROS precluded a caspase-dependent response, which was independently confirmed by the fact that none of the cells showed any additional signs of apoptosis when analyzed. Moreover, all these cells exhibited extremely low forward scatter, consistent damaged cellular debris, rather than whole cells, perhaps as a consequence of necrosis. These data strongly suggest that the presence of glucose in the growth medium is necessary for any cadmium induced apoptotic response in yeast cells.

(A) WT cells (BY4741)

(B) $\Delta YCA1$ cells

(C) WT cells (BY4741)

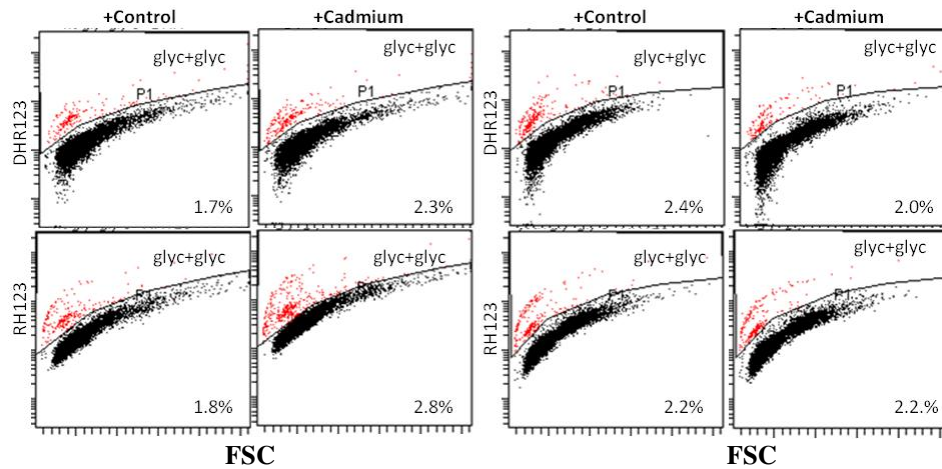
(D) $\Delta YCA1$ 

Figure 11: Cadmium is not able to induce mitochondrial membrane hyperpolarization and enhanced ROS levels when glucose is replaced with glycerol

Cells were incubated in the presence of $30\mu\text{M Cd}(\text{NO}_3)_2$ for 1h and then recovered in fresh medium for 3h. Heterogeneous production of intracellular ROS and changes in the mitochondrial membrane potential as a consequence of +Cd treatment were determined using DHR123 and RH123 dyes with respective controls as mentioned before. (A) & (B) Neither WT or $\Delta YCA1$ mutant cells showed an apoptotic response to the presence of cadmium when glucose is replaced with glycerol (C) & (D) Both WT and mutant $\Delta YCA1$ cells exhibited some amount of ROS and membrane hyperpolarization when exposed to Cd in presence of glycerol * **Note: Gluc+Glyc indicate the presence of glucose during 1h of metal exposure and glycerol during 3h recovery period (C) & (D) Glyc+Glyc indicate the presence of glycerol during 1h of metal exposure and also during 3h recovery period**

Having undertaken a series of ROS analyses to determine the importance of glucose in the apoptotic response of yeast to cadmium exposure, a similar series of experiments were undertaken to ascertain the importance of glucose in the apoptotic response of cells to a redox-active stressor, such as copper. As with cadmium, increased levels of ROS and increased mitochondrial membrane hyper-polarization have already been demonstrated in cells treated with 8mM Cu for 1h and incubated in YEPD medium deprived of copper for 3h (Nargund, 2010). Unlike cadmium, copper is capable of generating ROS directly -through Fenton or Harber-Weiss reactions (Stohs and Bagchi 1995; Valko, Morris et al. 2005). Consequently, as had been done for cadmium, the apoptotic response of cells to copper was determined using dihydroethidium (DHE) to measure the intensity of ROS generated within the Cu treated cells, and RH123 to assay the presence of any mitochondrial membrane hyperpolarization.

The extent to which ROS were produced in cells treated with copper (Fig **12A**) is seen to be far in excess of any produced by cells exposed to cadmium (Fig **10A**) in which approximately 20-25% of cells demonstrated a significantly heightened levels of ROS and mitochondrial membrane hyperpolarization.

These results were not too surprising, as Copper (Cu) belongs to a redox-active group of metals that has the capacity of generate ROS within the cell, directly through Fenton or Harber-Weiss reactions (Shanmuganathan, Avery et al. 2004; Liang and Zhou 2007; Nargund 2010). Thus, the ROS generated within the cell can be formed directly through the chemical activities of copper to produce free radicals, through hyperpolarized membranes that result in the increase in oxidative stress leading to reduction in membrane potential and breaking down of mitochondrial fragmentation, or a combination of both, with the consequences of each perpetuating the other.

(A) WT cells (BY4741)

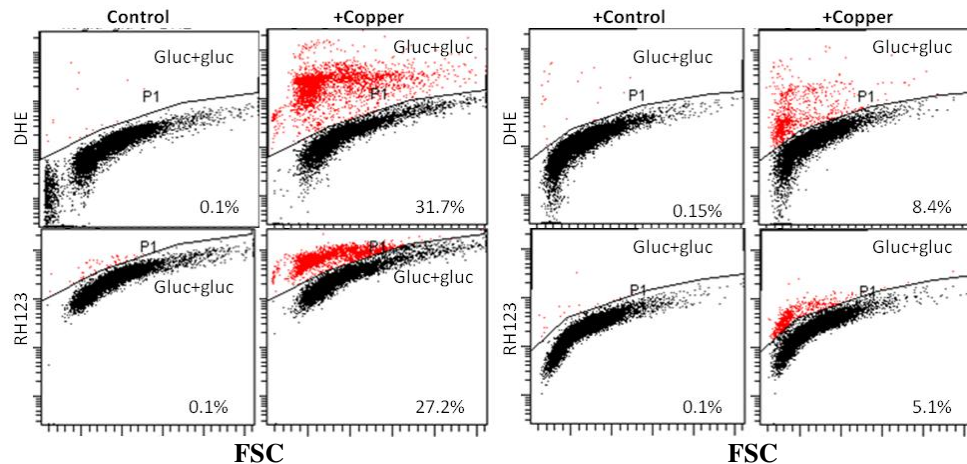
(B) $\Delta YCA1$ cells

Figure 12: Copper induced mitochondrial membrane hyperpolarization and enhanced ROS levels in presence of glucose

Cells were induced with 8mM $\text{Cu}(\text{NO}_3)_2$ for 1h and then recovered in fresh glucose containing (YEPD) medium for 3h with no cadmium present. Heterogeneous production of intracellular ROS was detected using the oxidant-sensitive fluorescent dye DHE. Cells were stained with DHE before 15min during the 3h post-incubation and fluorescence was analyzed with flow cytometer. Control cells were treated in exactly the same way as the +Cu treated cells by excluding Cu. Changes in the mitochondrial membrane potential as a consequence of Cu treatment were determined following staining with RH123. The cells that were incubated in the absence or presence of Cu were analyzed using flow cytometer. (A) & (B) WT cells did exhibit a large amount of ROS and membrane hyperpolarization because of Cu being redox-active in nature. YCA1 mutant cells also did exhibited ROS and membrane hyperpolarization but at lower levels compared to WT. **Note: Gluc+Gluc indicate the presence of glucose during 1h of metal exposure and also during 3h recovery period**

That these different mechanisms are potentially both responsible for generating ROS appears to be borne out by the number of cells exhibiting heightened levels of intracellular ROS and mitochondrial damage in the YCA1 mutant cells (Fig **12B**). The significant decrease in the number of YCA1 mutant cells oxidative stress (8.4% vs. 31.7% and 5.1 vs. 27.2%, respectively) suggests that at least two mechanisms are involved, one Yca1-dependent and the other Yca1-independent.

Intriguingly, when glucose is removed or replaced by glycerol during the 3 h post metal-exposure refractory period (Fig. 13 A & C), the number of WT cells that exhibit heightened ROS and mitochondrial membrane perturbations is reduced up to four-fold in comparison to cells exposed to copper in the presence of glucose for the duration of the analysis (Fig. 12A). Furthermore, the number of YCA1 mutant cells experiencing heightened ROS and damaged mitochondrial membranes when glucose is removed or replaced by glycerol in the refractory phase of the assay is actually 1.5 to 2-fold higher than it is WT (Fig. 13). These results would indicate that the absence of glucose (not merely the absence of a carbon source) has a significant influence on the ability of yeast cells to counteract the oxidative stress of the YCA1-independent production of ROS within the cells.

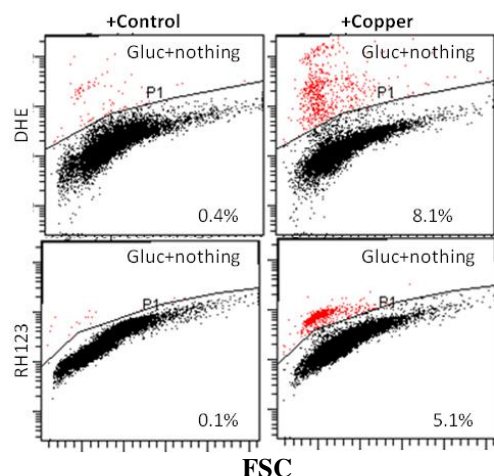
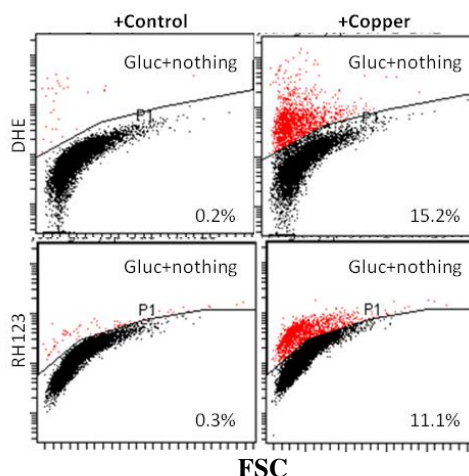
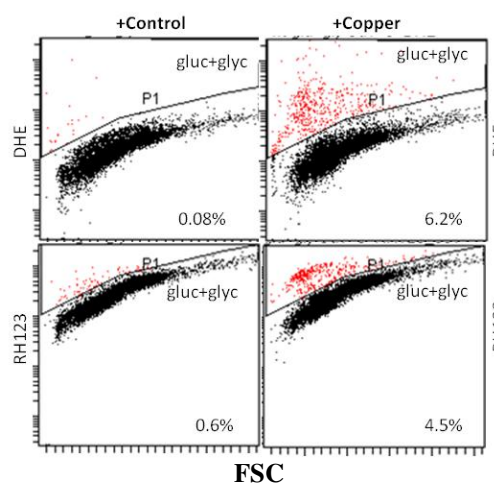
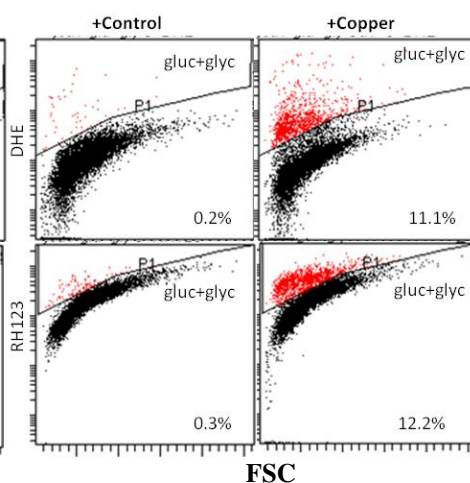
(A) WT cells (BY4741)**(B) $\Delta YCA1$ cells****(C) WT cells (BY4741)****(D) $\Delta YCA1$ cells**

Figure 13: Copper is still able to induce mitochondrial membrane hyperpolarization and enhanced ROS levels when glucose is not present during recovery period

Cells were induced with 8mM $\text{Cu}(\text{NO}_3)_2$ for 1h in the presence of glucose(gluc) then recovered in fresh medium for 3h. Heterogeneous production of intracellular ROS and changes in the mitochondrial membrane potential as a consequence of Cu treatment were determined using DHE and RH123 dyes as mentioned previously. (A) & (B) Reduced levels of ROS and membrane hyperpolarization was observed in WT than mutant YCA1 when glucose is removed (nothing) during the refractory period (C) & (D) Similar amount of cells exhibited apoptotic response when glycerol (glyc) is introduced into medium during the post-recovery period. *Note: (A) & (B) Gluc+nothing indicate the presence of glucose during 1h of metal exposure and no carbon source during 3h recovery period. (C) & (D) Gluc+Glyc indicate the presence of glucose during 1h of metal exposure and glycerol during 3h recovery period

Finally, when glucose is replaced entirely by glycerol in all phases of the process (Fig. 14A & B), the number of WT and Δ YCA1 cells experiencing heightened levels of ROS and mitochondrial hyperpolarization drops precipitously when compared to those exposed to glucose (Fig. 12 A & B). Moreover, the ROS profiles for both WT and Δ YCA1 cells are the same, and appear to be very similar to the ROS profiles of cells exposed to cadmium in the absence of glucose (Fig.11).

The data indicates that cells treated with copper also require an active carbon source for inducing apoptosis (i.e. in first hour of metal exposure) but may not require during the recovery period (i.e. in 3h recovery period where cells exhibit discernible effects caused due to metals). But YCA1 mutant cells exhibiting more amounts of ROS than WT in the absence of glucose during the refractory period, suggest that yeast caspase may be necessary for cells to overcome the stress. Thus these results evidently show that the presence of glucose and Yca1p is also necessary for cells treated with copper.

Though the purpose of choosing two different types of metal was to understand the mechanisms that underlie metal toxicity, we found that both the metals require glucose in first hour of metal exposure for yeast cells to undergo apoptosis. These findings suggest that these metals possess different pathways while exhibiting cellular responses, in which copper is able to generate ROS more than cadmium. The reason for more ROS is due to that copper being a redox-active is able to generate ROS by itself unlike Cd.

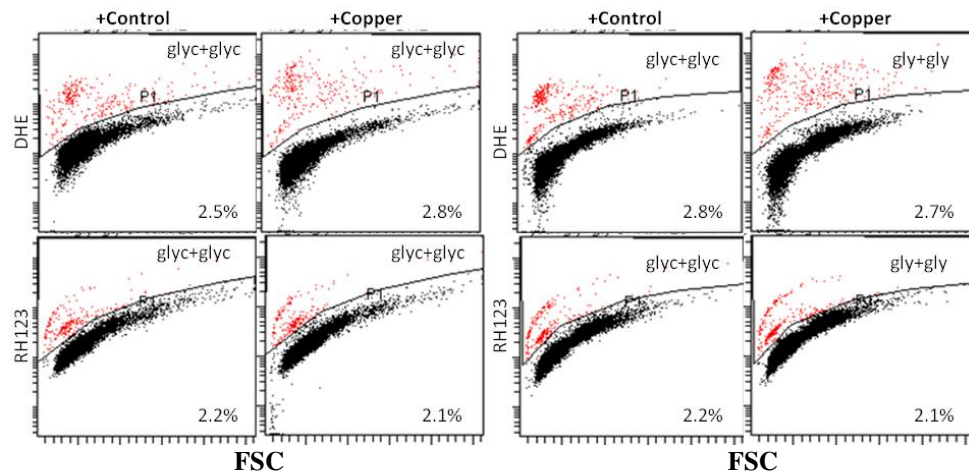
(A) WT cells (BY4741)**(B) $\Delta YCA1$ cells**

Figure 14: Copper is not able to induce mitochondrial membrane hyperpolarization and enhanced ROS levels when glucose is replaced with glycerol.

Cells were grown in glycerol containing medium YEPG and incubated in the presence of 8mM Cu (NO₃)₂ for 1h then recovered in fresh glycerol containing (YEPG) medium for 3h.

Heterogeneous production of intracellular ROS and changes in the mitochondrial membrane potential as a consequence of Cu treatment were determined using DHE and RH123 dyes as mentioned previously. (A) & (B) Neither WT nor YCA1 mutant cells exhibited apoptotic responses when cells incubated in glycerol (glyc) containing medium during 1h Cu exposure.

*Note: Glyc+Glyc indicate the presence of glycerol during 1h of metal exposure and also during 3h recovery period

FTIR Results and Discussion:

Figure 15 represents a typical spectrum from yeast cells. This spectrum looks similar to the spectra obtained from different cell types such as T-cells, leukemia cells, lymphocytes, etc., (Jamin, Miller et al. 2003; Silvia Gaudenzi 2004; Toro 2004; Zelig, Kapelushnik et al. 2009). Band assignments were based on previous FTIR studies on cells in the region from 1800 - 800 cm^{-1} represent a “fingerprint region” for studying cellular activities, since the most prominent biochemical changes that takes place in cells were observed in that region in response to uv-radiation or chemicals like etoposide, actinomycin D, $\text{Cd}(\text{Cl})_2$, etc., (Liu and Mantsch 2001; Gasparri and Muzio 2003; Silvia Gaudenzi 2004). The change in peaks at 1660 cm^{-1} and 1541 cm^{-1} were considered as the changes caused by the vibrations among the amide groups namely amide I and amide II bands. Also the peaks difference at 1465 cm^{-1} and 1415 cm^{-1} were caused by the bending motions in methylene groups that are arranged both symmetrically and assymmetrically. Similarly the band differences observed at 1453 cm^{-1} was considered to have present specifically due to asymmetrically arranged methylene groups. The bands present at ~1400 cm^{-1} corresponds to symmetric vibrations of bonds in methylene group and was also due to carboxylate group of fatty acids and aminoacid groups. The change in bands at 1237 cm^{-1} corresponds to phosphate stretching arranged asymmetrically and found in phosphodiester groups of nucleic acids. Also the band differences observed at 1080 cm^{-1} corresponds to multiple groups and bonds so called multicomponent band. It involves C-O, C-O-P, symmetric stretchching of bonds present in phosphate group each corresponds to sugars, phosphorylated lipids, phosphodiester groups of nucliec acids respectively along with some proteins and other macromolecules (Jamin, Miller et al. 2003; Silvia Gaudenzi 2004).

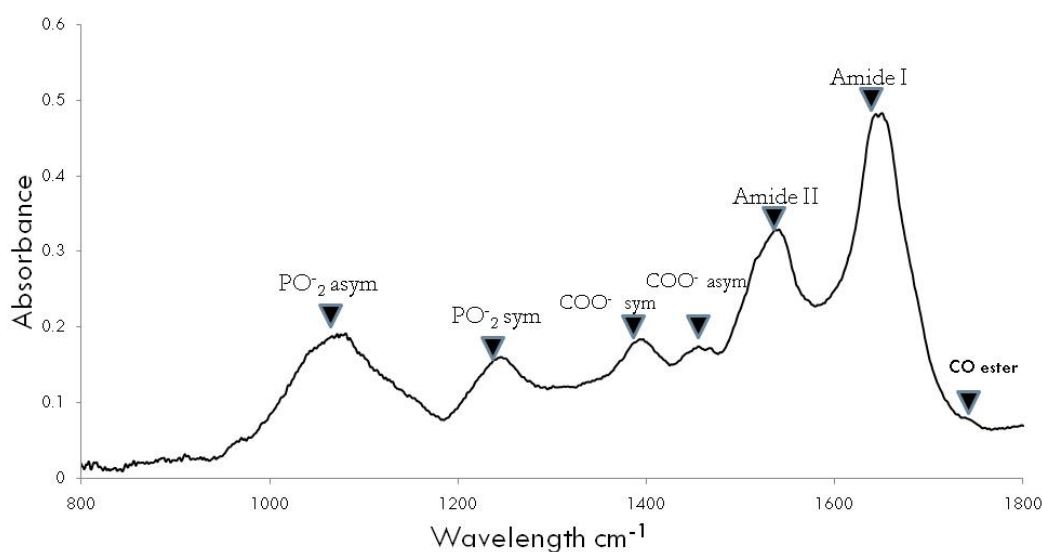


Figure 15 : Typical spectrum produced in *Saccharomyces cerevisiae* showing the region from 1800-800cm⁻¹

IR spectra of yeast cells incubated with 30μM Cd(NO₃)₂ for 3h were obtained from 3 different days samples. Two distinct types of spectra were observed that corresponds to WT Cd treated and WT untreated(no chemical added to the flask containing cells) . Figure 16 shows the raw data obtained at 60min, 120min and 180min. The spectra of the cells that were treated with Cd show differences in the peak heights particularly more at ~1040cm⁻¹ as we go from 60min to 180min. Thus, the spectral changes are consistent with the findings of Nargund et al (2008, 2010) in that cells need 3-4h of time after the initial metal exposure for any metabolic responses to be observed. Even so, the fact that some FTIR can be demonstrated within 1 h of exposure does represent the first time any changes in cellular function can be demonstrated for metal exposure in yeast.

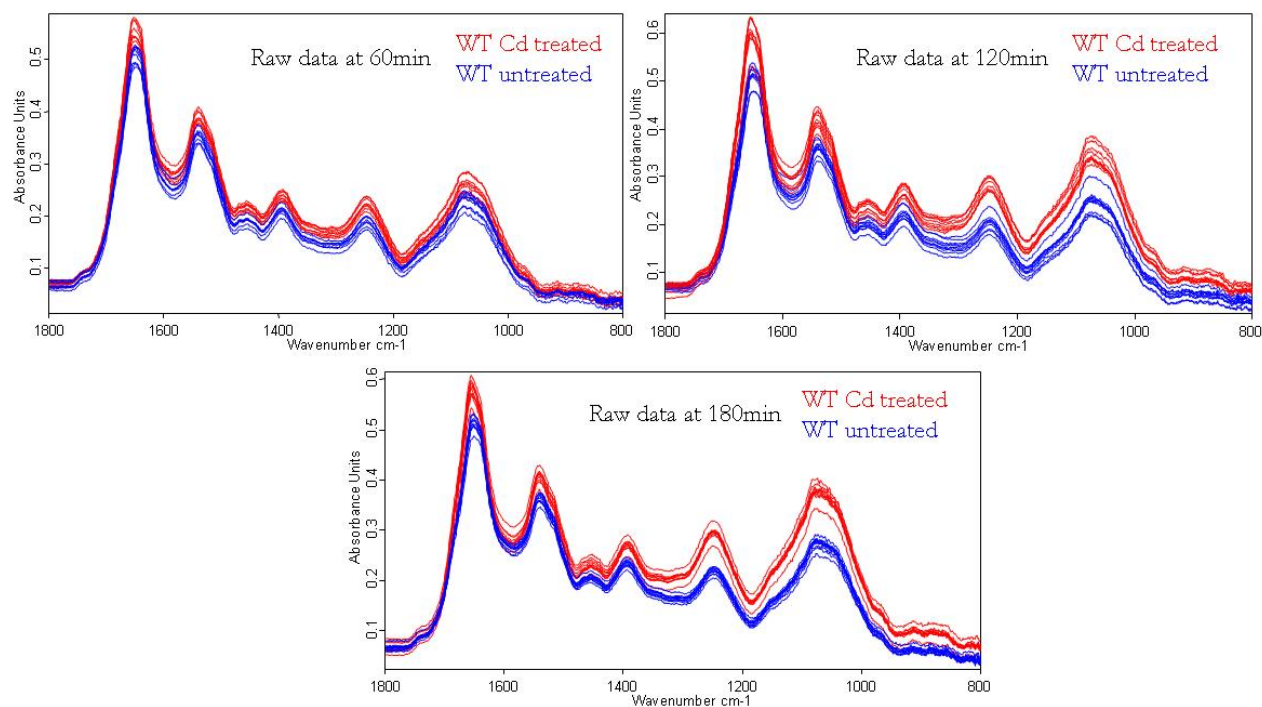
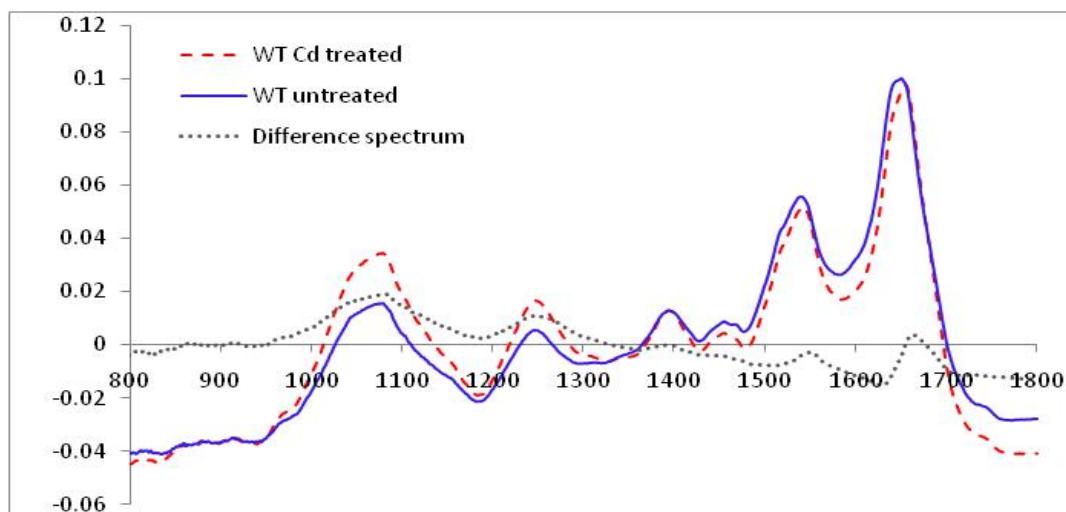


Figure 16: Raw data shown for WT cells that are Cd treated and untreated. Red lines indicate as WT Cd treated and blue lines as WT untreated. Each cell type that was spreaded on Low e glass slide were scanned around ~8-12 times for each experiment. ***Note: The order of X-axis has been changed from 1800-800cm⁻¹ in this figure**

After obtaining raw data, spectra were baseline corrected and the area between 800 and 1800cm⁻¹ was normalized using OPUS software. The average spectrum (Fig. 17) for each assay corresponds to spectra derived from different cell types on three different days data that has ~8 - 12 scans per analysis. While samples for each set of cells were taken at 60 min, 120min and 180 min after metal exposure, to allow for the best interpretation of FTIR spectral changes, only samples relating to cells at 180min after metal exposure were analyzed further. This analysis included a determination of a second derivative spectra from normalized FTIR values, so as to get a better understanding of changes in these spectra. In this way one can make sure that the changes that are visible in the normal spectra are for real and not any artifact.

(A) Normalized Average Spectra with difference spectrum



(B) Second derivative spectra

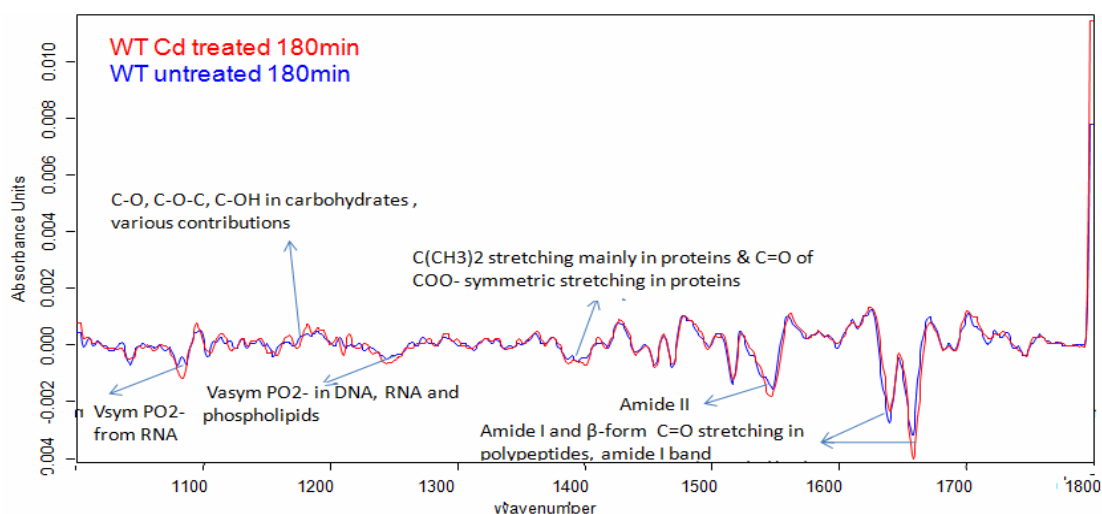


Figure 17: Spectral comparison between Wt Cd treated and Untreated after 3h treatment
(A) FTIR spectra obtained for Wt Cd treated (red) and untreated cells (blue) and corresponding difference spectrum (grey) after exposing to Cd for 3h. Spectra represents the average of 3 day experiments performed. **(B)** Second derivative spectra was obtained using OPUS software. It also elucidates the changes in spectra particularly at specific regions.

The FTIR spectra of Cd treated cells and untreated cells (Fig 17) were analyzed with the help of previous FTIR studies conducted on different cell types in apoptosis conditions. The positive bands from $1340\text{-}980\text{cm}^{-1}$ in the IR difference spectrum shown in Fig 17A (dotted lines in grey) indicate that the DNA content has been increased, while the negative bands from $1760\text{-}1380\text{cm}^{-1}$ indicate that the proteins are being damaged. In fig 17B, the obtained second derivative spectra clearly gives us the information about various biochemical changes that are taking place at particular wavelengths.

As a control, experiments were also performed on YCA1 mutant cell type. When compared with WT spectra, the spectra of YCA1 mutant, both were seen to exhibit similar pattern of cellular responses to the presence of metal, although the effects on the FTIR spectra for the YCA1 cells was seen to be significantly muted (Fig. 18). The YCA1 mutant cells do not undergo apoptosis when treated with Cd because of the fact that it is deficient in yeast capase (Yca1p) which is involved in triggering apoptosis in yeast (Nargund, 2008).

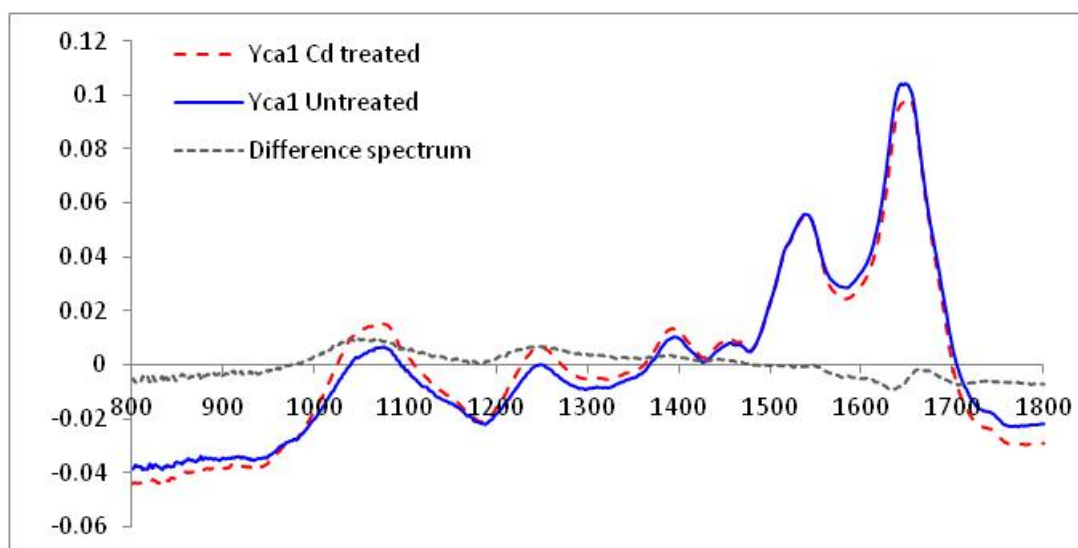


Figure 18: Spectral Comparison between Cd treated and Untreated cell population in YCA1 mutant: FTIR spectra obtained for treated (red) and untreated (blue) cells along with the difference spectrum (grey dotted lines). Spectra represents the average of 3 day experiments performed.

Although the results indicate the same band patterns changes at the specific regions of FTIR spectra, it is important to note that with Wild-Type cells, at least, these data were obtained from a heterogeneous population of cells. As such, it was important to determine whether these changes are a generalized cellular response of yeast or whether these metabolic changes are specific to the sub-population of yeast cells that were undergoing apoptosis. So we tried to obtain pure apoptotic cells from a heterogeneous population of cells by performing sorting experiments. The cells were stained with DHE dye after being exposed to Cd for 1h and further recovered in fresh YEPD medium for 3h devoid of cadmium. Cells were sorted into apoptotic and non-apoptotic populations, using a FACS Aria cell sorter from BD, and then analyzed under FTIR, defined previously. The spectra that were obtained for both sub populations of cells (Fig 19) were quite different to each other, with the pre-apoptotic sub population of cells showing a substantially different absorbance profile to that of the non-apoptotic sub population. Intriguingly, the trends in the FTIR band for pre-apoptotic cells was in remarkable agreement with the FTIR profile gathered for other cells going through apoptotic responses, such as CEM cell FTIR profile obtained by Liu et. al (Fig 20).

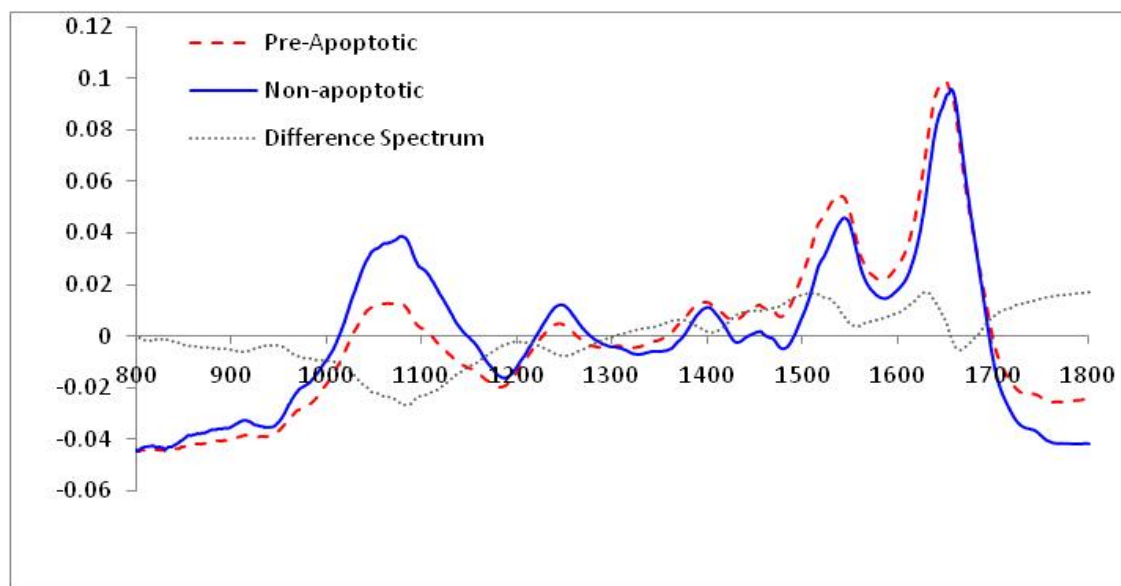


Figure 19: Spectral Comparison between apoptotic and non-apoptotic population after sorting

FTIR spectra obtained for apoptotic (red) and non-apoptotic (blue) cells along with the difference spectrum (grey). Spectra represents the average of 3 day experiments performed.

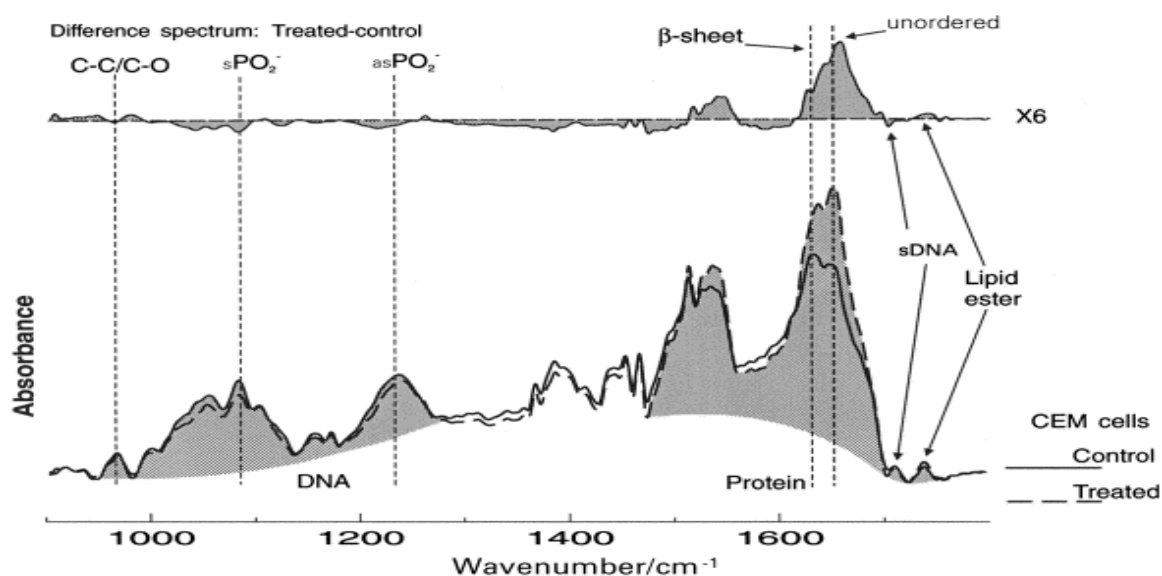


Figure 20: Representative IR spectra of CEM cells before and after treatment (24 h) with etoposide (bottom), and IR difference spectrum between control and the apoptotic (etoposide-treated) CEM cells (Liu and Mantsch 2001)

The spectral pattern of the pre-apoptotic WT yeast cells (Fig 19), showed a shift in peaks 1660 and 1540 cm^{-1} corresponding to the formation/breakage of amide I and amide II bonds, along with an increase in the absorbance peak heights at these wave numbers. Based on studies done by Gasparri et. al, the components of amide I and amide II band gives information about the α -helix, random-coil, β - sheets and β -turn structures ranging from 1700-1500 cm^{-1} (Gasparri and Muzio 2003). The shift at these regions is due to the fact that there could be structural modifications in α -helix and β -sheets during apoptosis condition that can be detected by FTIR. The increase in absorbance in amide I and amide II regions indicates that the overall protein structure in the non-apoptotic cells consists primarily of parallel β -sheet constituents, whereas apoptotic cells have a relatively high proportion of unordered proteins (Gasparri and Muzio 2003).

The other factor that is considered mostly was the DNA absorbance parameter, because of already established fact that DNA absorbs less IR during apoptosis. It remained consistent with our data also when compared between pre-apoptotic and non-apoptotic cells because there is a huge peak drop in pre-apoptotic cells spectra with respect to absorbance at 1080 cm^{-1} . There is a also decreased absorbance of the asymmetric and symmetric phosphate stretching vibrations at 1240 cm^{-1} and 1080 cm^{-1} . The band at 1220 cm^{-1} corresponds to the asymmetric phosphate stretching mode of the fully hydrogen bonded PO_2^- group. These changes in FTIR pattern might be due to several proposed hypothesis, that non-Beer Lambert's law absorption might be one of the reasons (Zelig, Kapelushnik et al. 2009).

By superimposing the FTIR profiles of the non-apoptotic cells (Fig 17) over the FTIR profiles of the whole cell populations that had been exposed to cadmium (Fig 19), it is obvious that all changes in FTIR spectra that are apparent in the whole population treated with cadmium

are entirely due to the cellular responses of the non-apoptotic cells, and that there are no changes in absorbance spectra that are overtly due to any pre-apoptotic cellular responses (Fig 21). And the reason could be that we are looking at mixed population which comprises of both cells undergoing apoptotic and cells that do not undergo apoptosis but affected by Cd. So the changes that we observed when cells treated with Cd is merely the response generated by the cells undergoing metal treatment and that are recovering. Thus the results of the study indicated that biochemical changes are taking place when cells were metal treated.

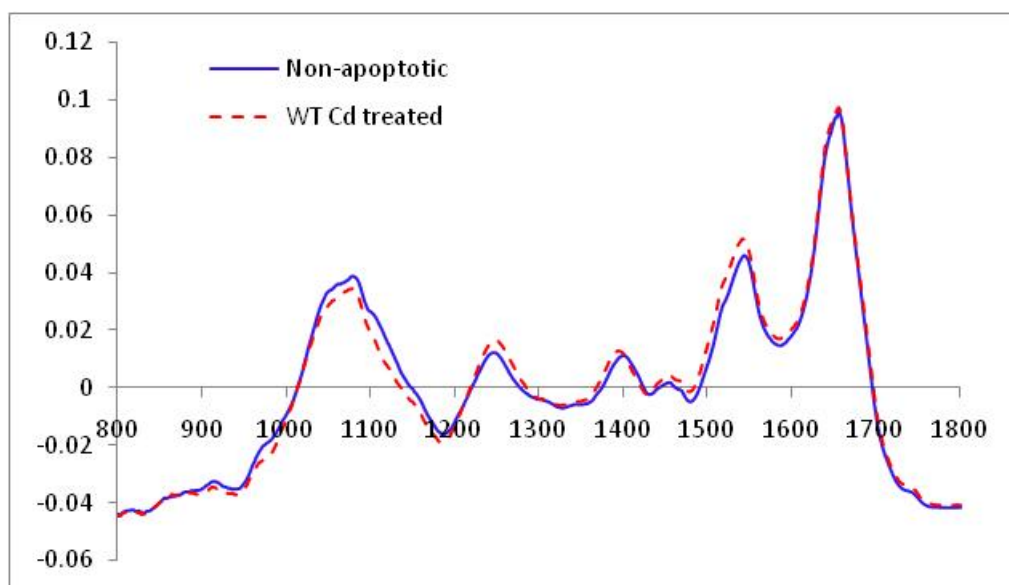


Figure 21: Spectral Comparison between Cd treated and non-apoptotic cell population in WT: FTIR spectra obtained for treated (red) and non-apoptotic (blue) cells. Spectra represents the average of 3 day experiments performed.

Conclusions:

The data from flow cytometry analysis suggests that when glucose is removed or replaced, Cd treated cells were not able to induce or exhibit apoptotic response suggesting that cells require active metabolism for apoptosis to be taken place. But upon Cu treatment, both WT and YCA1 mutant cells were able to show ROS and mitochondrial hyperpolarization even when glucose is removed or replaced during the 3h refractory period. This confirms that both metals are inherently different in nature and they take different routes in generating cellular responses. The data also indicate an absolute requirement for glucose (not merely a carbon source) because cells were not able to induce apoptosis upon metal exposure when glucose is replaced with glycerol through the entire process. This confirms that glucose is absolutely necessary for cells to induce any kind of cellular response in response to metals.

The data also suggest that both copper and cadmium generate different types of ROS, in which copper generate ROS independent of yeast caspase, unlike Cd. It may be due to the fact that copper being a redox-active metal, generates ROS by itself where cadmium does that indirectly and depends on the activation of yeast caspase. Thus our results do not turf off the fact that yeast cells also require Yca1p to undergo metal-induced apoptosis.

The FTIR data suggests IR spectroscopy can be used for monitoring changes that are being taken place in response to a stimulus that leads to apoptosis. In particular, the behavior of amide bonds at 1670 and 1540 cm^{-1} and that of DNA absorbance parameter at 1080 cm^{-1} in apoptotic cells represents a high correlation between our data and previous studies data as shown. The quantitative analysis may help us in drawing conclusions specific to the changes that were observed qualitatively. The data of both WT and mutant YCA1 cells exposed to cadmium for 3hrs suggest that the cells were being affected biochemically. In summary, our results do

support our hypothesis that FTIR is able to monitor the events triggered in yeast upon Cd treatment.

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