Exogenous iron causes precipitation that precedes iron mediated cell death in yeast

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by

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Abstract

The essential micronutrient iron is associated with two forms of human pathophysiologies; namely anemia because of inadequate iron and toxicity because of excess iron. Its ability to gain and lose electrons as it interconverts between ferrous (Fe^{2+}) and ferric (Fe^{3+}) forms makes it indispensable as an electron carrier in many cellular processes. This same property makes iron toxic to the cell given that free electrons can react with cellular constituents to produce highly toxic Reactive Oxygen Species (ROS). The prevailing model of iron toxicity is that excess iron enters the cell and induces cell death by increasing the levels of ROS. The Greenwood lab uses the yeast *Saccharomyces cerevisiae* as a simplified model to study cellular responses to excess iron. Previous results show that excess extracellular iron does not enter cells and is associated with the formation of an insoluble precipitate with the yeast growth media. This research is focused on the hypothesis that exogenous iron causes cell death by precipitating with an essential component of yeast media, leading to nutrient depletion. Using spot growth assays, we show that iron is a dose-dependent inhibitor of yeast growth. No growth inhibition was observed at concentrations below 5mM iron, half-maximal growth inhibition was observed with 7mM, while complete growth inhibition occurred at a concentration of 8mM $FeCl_3$. In an analogous fashion, the iron mediated accumulation of precipitation in yeast growth media was also found to increase in a dose dependent manner. Pronounced precipitation was observed at concentrations of iron as low as 0.4mM, with half-maximal precipitation occurring at 4mM, while maximum precipitation required 20mM $FeCl_3$. These observations demonstrate that iron mediated accumulation of a precipitate in YNB yeast growth media is an event that precedes the negative effects of iron on yeast cell growth. This suggests that the formation and accumulation of a precipitate serves to mediate the toxic effects of excess iron. Analysis of the chemical composition of yeast growth media suggests that the phosphate anion (PO_4^{3-}) is likely responsible for the observed precipitate. This is based on two separate considerations: first, PO_4^{3-} is present at sufficiently high concentrations to account for the formation of 4.5mg of precipitate from 1 ml of YNB yeast media, and second, the PO_4^{3-} anion is highly insoluble with Fe^{3+}, with a Ksp value for iron phosphate ($FePO_4$) of 9.91×10^{-16} (25ºC). In conclusion, the

results showing that iron mediated precipitation precedes iron mediated growth inhibition supports the hypothesis that iron toxicity is due to the depletion of phosphate, an essential micronutrient. Support for this comes from a previous study showing that depletion of phosphorus leads to the induction of apoptosis like cell death.

Résumé

Le fer, micronutriment essentiel, est associé à deux formes de physiopathologies humaines ; l'anémie en raison d'une carence en fer et la toxicité en raison d'un excès de fer. Sa capacité à gagner et à perdre des électrons lors de l'interconversion entre les formes ferreuse (Fe^{2+}) et ferrique (Fe^{3+}) le rend indispensable en tant que porteur d'électrons dans de nombreux processus cellulaires. Cette même propriété rend le fer toxique pour la cellule étant donné que les électrons libres peuvent réagir avec les constituants cellulaires pour produire des espèces réactives de l'oxygène (ROS) hautement toxiques. Le modèle dominant de toxicité ferreuse est que l'excès de fer pénètre dans la cellule et induit la mort cellulaire en augmentant les niveaux de ROS. Nous utilisons la levure *Saccharomyces cerevisiae* comme modèle simplifié pour étudier les réponses cellulaires à l'excès de fer. Nos résultats précédents montrent que l'excès de fer extracellulaire ne pénètre pas dans les cellules et est associé à la formation d'un précipité insoluble avec les milieux de croissance de levure. Mes recherches portent sur l'hypothèse selon laquelle le fer exogène provoque la mort cellulaire en précipitant avec un composant essentiel des milieux de levure, entraînant un épuisement de nutriments. En utilisant des tests de croissance, nous montrons que le fer est un inhibiteur dose-dépendant de la croissance des levures. Aucune inhibition de la croissance n'a été observée à des concentrations inférieures à 5 mM de fer, une inhibition de croissance semi-maximale a été observée avec 7 mM, tandis qu'une inhibition de croissance complète s'est produite à une concentration de 8 mM de $FeCl_3$. De manière analogue, l'accumulation de précipitation médiée par le fer dans les milieux de croissance de levure s'est également avérée a augmenter de manière dose-dépendante. Des précipitations prononcées ont été observées à des concentrations de fer aussi faibles que 0,4 mM, avec des précipitations semi-maximales se produisant à 4 mM, tandis que les précipitations maximales nécessitaient 20 mM de $FeCl_3$. Ces observations démontrent que l'accumulation médiée par le fer d'un précipité dans les milieux de croissance de levure YNB est un événement qui précède les effets négatifs du fer sur la croissance des cellules de levure. Cela suggère que la formation et l'accumulation d'un précipité servent à médier les effets toxiques de l'excès de fer. L'analyse de la composition chimique du milieu de croissance de levure suggère

que l'anion phosphate (PO_4^{3-}) est probablement responsable du précipité observé. Ceci est basé sur deux considérations distinctes : premièrement, PO_4^{3-} est présent à des concentrations suffisamment élevées pour expliquer la formation de 4,5 mg de précipité à partir de 1 ml de milieu de levure YNB, et deuxièmement, l'anion PO_4^{3-} est hautement insoluble avec Fe^{3+}, avec une valeur Ksp pour le phosphate de fer ($FePO_4$) de $9{,}91 \times 10^{-16}$ (25ºC). En conclusion, nos résultats montrant que la précipitation médiée par le fer précède l'inhibition de la croissance médiée par le fer soutient notre hypothèse selon laquelle la toxicité ferreuse est due à l'épuisement du phosphate, un micronutriment essentiel. Cette est aussi supporté des études précédente montrant que l'épuisement du phosphore conduit à l'induction de la mort cellulaire apoptototique.

Co-Authorship

I am the first author of the manuscript integrated in this thesis. Along with David Zhou as second author, a PhD student in Anatomy and Cell Biology at McGill University, in Montreal, Quebec. It may undergo some minor organizational changes but it is in preparation to be submitted for publication in PLOS One.

As a first author, I am responsible for the creation of the spot growth assays, precipitation assays, and solubility assays used throughout the study and labour involved in producing all figures in the manuscript as well as in this thesis.

David Zhou is responsible for the work involved in producing Figure 3.4 as well as helping with final revisions of all figures in the manuscript.

Rawan Eid is a former PhD student in Dr. Greenwood's laboratory. She was responsible for the concepts and work involved in the initial stages of this project.

Craig Mandato is responsible for the co-supervision of David Zhou at McGill University.

Michael T. Greenwood developed the concept of the study and all of the listed authors contributed to its design and assisted in the preparation and revision of the manuscript.

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Abbreviations

Acronym/ Abbreviation	Description
ANOVA	Analysis of variance
cAMP	Cyclic adenosine monophosphate
CNS	Central nervous system
CO	Carbon monoxide
Cu	Copper
$CuSO_4$	Copper (II) sulphate
DMEM	Dulbecco's modified eagle medium
DMT1	Divalent metal transporter 1
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ETC	Electron transport chain
Fe	Iron
$FeCl_3$	Iron (III) chloride
$FePO_4$	Iron (III) phosphate
$FeSO_4$	Iron (II) sulphate
GAL	Galactose
Gal/Raf+++	Galactose raffinose media with leucine, lysine, histidine
GI	Gastrointestinal
H_2O_2	Hydrogen peroxide
HH	Hereditary hemochromatosis
ID	Iron deficiency
IDA	Iron deficiency anemia
ISC	Iron-sulphur clusters
N	Nitrogen
NO	Nitric oxide
O_2	Oxygen
$O_2^{\bullet-}$	Superoxide radial ion
$OH^{\bullet-}$	Hydroxide free radical
PCD	Programmed cell death
Raf	Raffinose
RBC	Red blood cells
ROS	Reactive oxygen species
S	Sulphur
S. cerevisiae	*Saccharomyces cerevisiae*
XPS	X-ray photoelectron spectroscopy

Chapter 1 – Introduction

Iron is an essential element for life, participating in a variety of cellular processes. Its essentiality, coupled to its scarcity in bioavailable forms, has required living organisms to develop mechanisms that ensure an adequate iron supply [1]. On the other hand, the versatility of iron as it interchanges between ionic states (Fe^{2+} and Fe^{3+}) can make it toxic due to the production of free radicals that can then damage cellular components and thus interfere with vital processes [2]. Thus all iron utilizing organisms have had to develop and maintain a complex network to regulate the uptake, excretion, and distribution of iron in order to limit toxicity [2]. Iron deficiency and excess iron overload are toxicity and health concerns, which affect individual cells as well as more complex systems such as organs in multicellular organisms [2].

The current model of iron toxicity is that excess iron enters the cell and induces cell death by increasing the levels of stress, also known as reactive oxygen species (ROS). We use the yeast *Saccharomyces cerevisiae* as a simplified model to study cellular responses to excess iron.

This specific research addresses questions regarding iron toxicity, and explores the different oxidation states and whether there is an effect on toxicity. In addition, to build on previous findings regarding iron toxicity and the formation of an unknown precipitate in the media [3], I investigate how the precipitate is forming and accumulating and whether the precipitate itself is a factor in cell death using both iron and copper chemical stresses on model yeast cells [3]. The hypothesis for this research is that exogenous iron mediated precipitation occurs before iron mediated cell death, and that iron induced cell death occurs due to depletion of an essential micronutrient in the growth media.

Chapter 2 – Literature Review

2.1 Elemental iron

2.1.1 Iron chemistry

Iron, atomic number 26, is an abundant atom, making up approximately 6% of the earths crust [1], [4]–[6]. It is one of several transition metals, including copper, zinc, manganese, and cobalt, that are essential metal ions for humans [1], [5], [7], [8]. Iron can exist in eight different oxidation states ranging from -2 to +6, but the 2+ and 3+ states are the most biologically relevant [1], [6]. Its ability to gain and lose electrons as it interconverts between ferrous (Fe^{2+}) and ferric (Fe^{3+}) forms is what makes it indispensable as an electron carrier in cellular processes [9].

Since iron is an important element for multiple biological processes, it is important to take into account the relative solubility for the oxidation states [10]. Ferric (Fe^{3+}) hydroxide is the predominant form of iron and has a solubility of 10^{-18} M in neutral solutions, which is far less soluble than ferrous (Fe^{2+}) iron salts which have solubilities in the 10^{-2} M range [4], [9], [11]. Thus, ferrous iron, being more soluble than ferric, is more prevalent in biological processes but much less accessible. Nevertheless, iron is continuously cycling and interconverting between the 2+ and 3+ states because of its oxidation and reduction in biological processes [1], [5].

Iron's capacity to exchange electrons in biological reactions is extraordinary but it can also be challenging. Iron is an intrinsic reactive oxygen species (ROS) producer and typical iron toxicity comes from the production of ROS [1], [9]. When one, or more, of its six ligand binding sites is unoccupied it can engage in reactions where only one electron is exchanged and free radicals are produced [1], [9]. Fe^{2+} reacts with oxygen to produce a superoxide radical ion ($O_2^{\bullet -}$) , which can further react to form oxygen (O_2) and hydrogen peroxide (H_2O_2). Additionally, it can also react with H_2O_2 to generate a highly reactive hydroxyl free radical [1]. The intermediates can be seen in Figure 2.1 below.

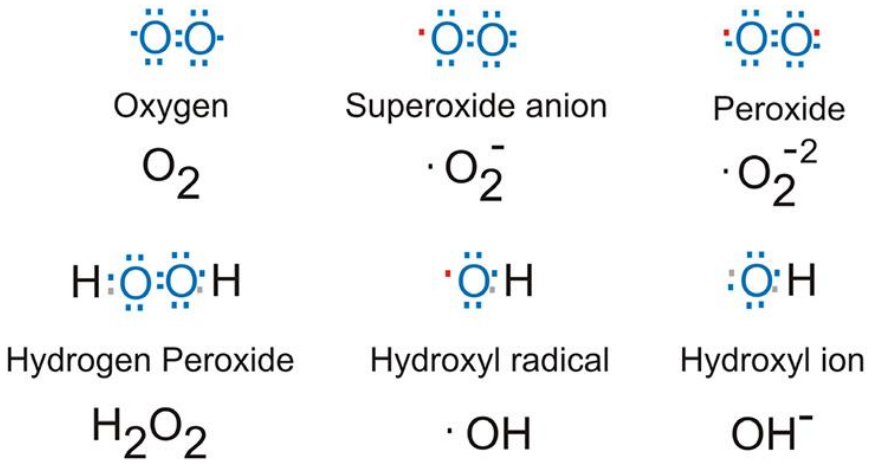


Figure 2.1. Reactive oxygen species intermediates and unpaired electrons.

The progression of ROS intermediates and their unpaired electrons. The single red electrons represent unpaired electrons. The unpaired electrons represent the toxic free radicals [12]**.** Image taken from P. Held, 2012.

Additionally, the generation of the free radicals can be explained through Fenton chemistry, using the Haber-Weiss and Fenton reactions [4], [13], [14]. The equations describing these processes are outlined below in Figure 2.2.

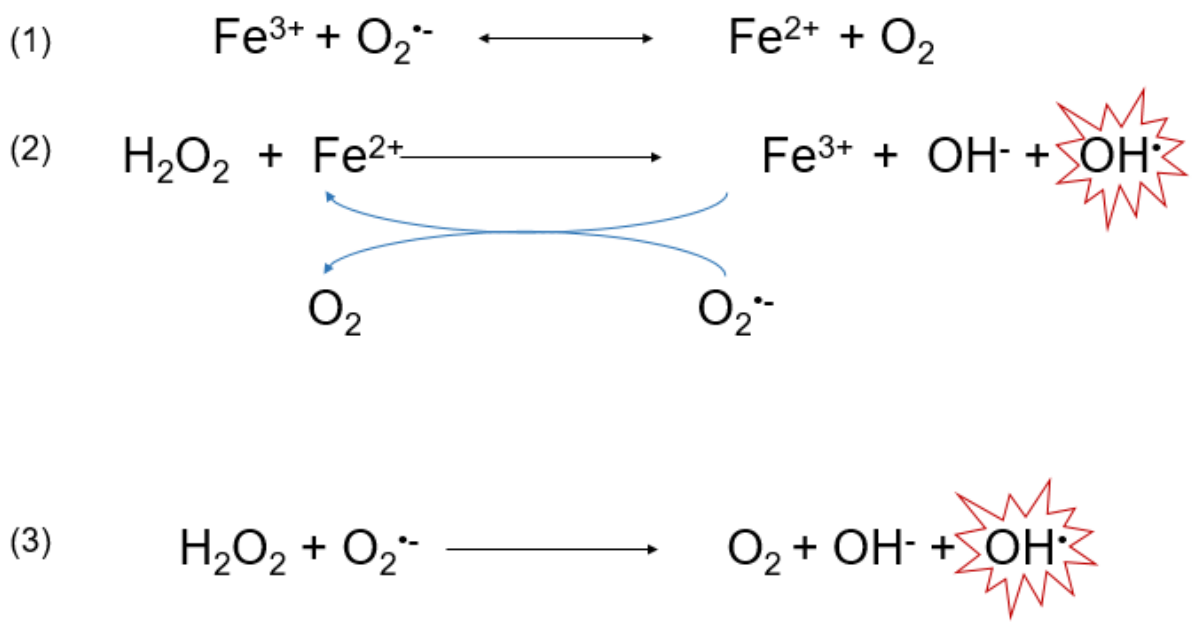


Figure 2.2. Fenton and Haber-Weiss reactions.

(1) The first equation of the Fenton reaction where ferric iron reacts with superoxide ion to form ferrous iron and oxygen [9]. (2) The final equation involved in the Fenton reaction which ferrous iron reacts with hydrogen peroxide to produce hydroxyl radical and hydroxyl ion [9]. (3) represents the net Haber-Weiss reaction [14]. All equations are iron-dependent.

The first set of equations, 1-2, are known as the Fenton reaction, while equation 3 is the net Haber-Weiss reaction. The Fenton reaction is the reaction of Fe^{2+} with hydrogen peroxide to produce a toxic hydroxyl free radical. The free iron and hydroxyl free radicals contribute to the production of ROS [14]. These reactions occur spontaneously in all aerobically growing iron containing cells [9].

In addition to ROS, another problem commonly encountered with iron is the scarcity of biologically available iron. Even though elemental iron is abundant, most forms are insoluble and not easily accessible biologically [9]. These conditions has led to the development of sophisticated biological mechanisms that serve to maintain iron homeostasis with the dual goal to obtain sufficient iron supply for essential cellular processes, while protecting from potential iron toxicity due to iron-mediated oxidative stress [4]. Every mammalian cell needs iron as a trace element in numerous biological processes; therefore, it is necessary to understand the complex nature of how iron could be toxic.

2.2 Iron in human health

Iron is important for human health and growth. As mentioned above, its biological role in mammalian organisms is in part attributable to its properties as a transition metal, which readily interconverts between different oxidation states [2]. This makes iron an essential prosthetic group for important cellular processes and electron transfer reaction [2]. Of importance for mammals and many other multicellular organisms is that iron's ability to interact with different forms of oxygen also allows it to carry out essential functions such as acting as an oxygen carrier in hemoglobin [15]. As an essential micronutrient, iron is associated with two main forms of human pathophysiologies; namely anemia because of inadequate iron and toxicity because of iron overload [9].

2.2.1 Micronutrients

Human nutrition provides the building blocks for all metabolic and physical functions [16]. Nutrition comes from the food we eat. Foods are a complex combination of compounds, known as nutrients that contribute to functions and metabolic processes [16], [17]. Nutrients can contribute biologically after they are consumed, as long as they are adequately separated by digestion [16]. Not all nutrients are available. Some are partly metabolized within the bowel or only partially absorbed, so less than 100% of the nutrients in a food are available to the consumer [16].

Nutrition is a critical element for normal growth and development, at a cellular, organ and whole-person level [16]. Persistent disturbances of nutrient metabolism and/or energy homeostasis, caused by either nutrient deficiency or excess, can induce consequences such as cellular stress leading to metabolic dysregulation and tissue damage [17]. Therefore, it is of the utmost importance to regulate and be consistent with food and nutrition for overall human health.

Nutrients may be categorized as macronutrients, which are proteins, fats, and carbohydrates [18]. These are needed in larger amounts and the benefits of consuming macronutrients are that they are the building blocks of cellular structures and energy substrates [17]. Some nutrients are needed at greater

quantities than others and at different stages of life [19]. Another type of nutrients are micronutrients, which include all vitamins, minerals and trace elements, including Vitamin A, D, and E, as well as the minerals, such as calcium, zinc, and iron [16], [17] . Micronutrients are needed only in very small amounts and are required for the proper function of vital proteins and enzymes [17]. Many in vivo roles of micronutrients are well known and established [20]. Much of the knowledge on the role of micronutrients have come from animal experiments where the effects of diets lacking one specific nutrient were examined. In humans, there are well known cases where individuals were specifically undernourished for different micronutrients that are not naturally synthesized in the body. For example, scurvy was a common condition for sailors on long journeys out to sea [21]–[23]. The symptoms were found to be diverse and included loss of teeth. All symptoms could be eliminated with the addition of citrus fruit or, more specifically, Vitamin C [21]–[23]. Later studies found that Vitamin C is required for growth and repair of tissues in all parts of the body, including helping the body absorb iron and producing collagen [21]–[23]. Thus, an understanding of the essential micronutrients is necessary, as they are vital for metabolic processes.

2.2.2 Iron's role in cells, as an essential micronutrient

Iron is an important cofactor required for a number of essential cellular functions, and hence is a vital micronutrient. Humans cannot synthesize it; therefore, exogenous sources, which can be absorbed, are necessary. Dietary iron is available in two forms, heme and non-heme [24]–[27]. Heme iron sources include hemoglobin and myoglobin, which come from the consumption of meat, poultry, and fish [24]–[28]. On the other hand, non-heme iron is acquired from cereals, legumes, fruits, and vegetables [24]–[28]. Heme iron has a much higher bioavailability than non-heme iron, 15-35% and 2-20% respectively [24]–[28]. The heme form of iron is easily absorbed because it is not influenced by the many dietary ligands in the diet [29]. It is directly taken up into the enterocytes by an absorption pathway that is different from non–heme-iron [29]. Heme-iron is also largely unaffected by the high pH of the upper small bowel, which renders some forms of inorganic iron insoluble [29]. This means that heme iron is absorbed more

efficiently than non-heme iron. The absorption of non-heme iron is strongly influenced by the presence of other food components, whereas the absorption of heme iron is not affected by dietary factors [24]–[28]. Although the bioavailability of non-heme iron is lower, the quantity available in our diets is significantly greater and thus is a greater contributor to iron nutrition than heme iron [24]–[28].

Iron is ingested from our diets and is tightly controlled and regulated by our body [9]. The necessary amount of iron required per day is approximately 8mg for adult men, 18mg for adult women, and 27 mg for pregnant adult women [4], [9], [25], [30]. Due to the fact that iron is very efficiently recycled and very little is lost, the amount of iron needed for daily replenishment is correspondingly low at 1-2mg per day; however, these values can vary [9]. The daily requirements of iron, or any vitamin or nutrient, is dependant on age, sex, and type of diet [25]. Vegetarians who consume a mostly plant-based diet need almost twice as much iron because the body does not absorb the non-heme iron found in plants [25]. For those who eat meat, poultry, or seafood, the daily recommended value of iron will be lower since the absorption of heme iron is higher [25]. Some major inhibitors of iron absorption are phytic acid, polyphenols, calcium, and peptides from partially digested proteins [24]. Conversely, some enhancers are ascorbic acid and muscle tissue which may reduce ferric iron to ferrous iron and bind it in soluble complexes which are available for absorption [24]. Ultimately, diet mediated absorption of iron reflects the needs of the body and ensures that iron concentration does not increase to levels that could lead to overload and potentially be toxic [9].

2.2.3 Iron metabolism

In the human body, iron exists as a complex form bound to proteins like heme, transferrin and ferritin, as well as other ions, mainly citrate [24]. The body requires iron mainly as a co-factor for a large number of different proteins. These proteins are required for many cellular processes like oxygen transport of hemoglobin and electron transfer and oxidation-reductions that occur in mitochondria, chloroplast and other electron transport chains [15], [24], [31]. Around two thirds of biological iron is found in hemoglobin present in circulating erythrocytes, 25% is readily available as an iron store which is mostly intracellular, 15% is bound to myoglobin and the remainder in a variety of enzymes involved in metabolism [24], [27].

Hemoglobin is one of the most well known examples of how iron is important for structure and function (Figure 2.3). The hemoglobin molecule consists of four subunits, each with its own alpha or beta polypeptide chain surrounding a prosthetic heme group [32]. The heme group consists of a ferrous iron ion connected to the four nitrogen (N) atoms of a porphyrin [33]. The prosthetic heme group is important for the binding and release of oxygen [32], [33]. The iron ion can also form additional bonds on its fifth and sixth sites, one on each side of the heme [33]. Oxygen binds reversibly to the ferrous iron ion in each heme group and assists with the binding and release of oxygen [32].

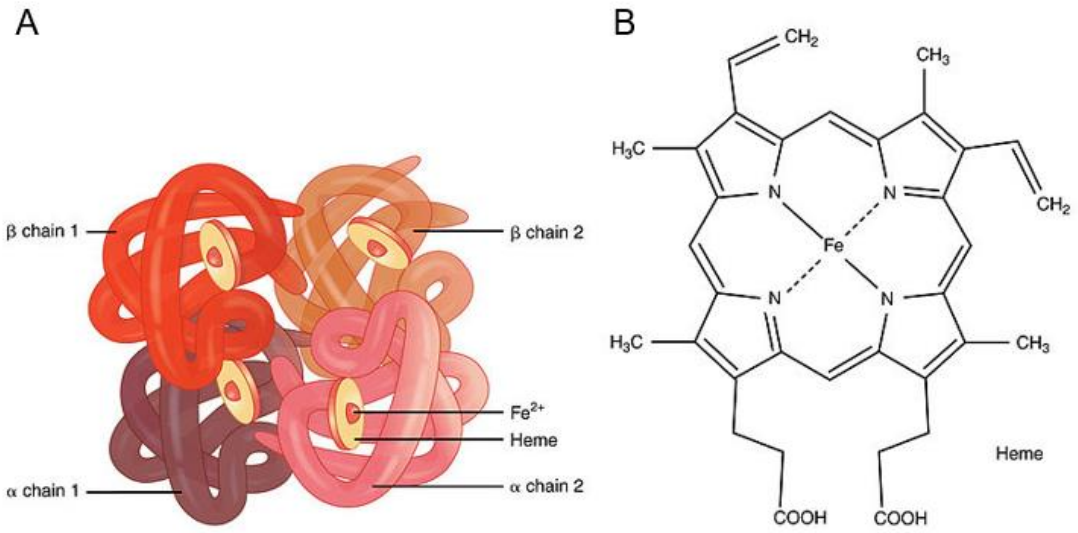


Figure 2.3. Structure of hemoglobin and prosthetic heme group.

(A) Hemoglobin is a structure that transports oxygen. The structure consists of four heme groups, surrounded by either an alpha or beta globin chain. There are two alpha and two beta chains. The heme group is a ringed molecule with a ferrous iron ion in the centre [32], [34]. (B) The prosthetic heme group within hemoglobin. The ferrous ion of the heme group is linked to the nitrogen (N) of a histidine, forming a ring. This ring is embedded in the polypeptide alpha or beta chain [32]. The prosthetic group allows binding and releasing of oxygen [34]**.** Image taken from Betts et al, 2013.

Another member of the globin family is myoglobin. It is found in the striated muscles of vertebrates, and is present in much lower concentrations in smooth muscle, endothelial and even tumour cells [23], [35]–[37]. The body uses its high affinity for oxygen as an oxygen storage protein in muscle [36]. Like

hemoglobin, it contains a heme prosthetic group that can reversibly bind to oxygen (Figure 2.4). This binding site can also function to bind other potential molecules like carbon monoxide, CO, and nitrogen oxide, NO [23], [35]–[37]. Thus, iron is a key element in biological functions as it can be in a prosthetic group which, when embedded in proteins can give them additional functions.

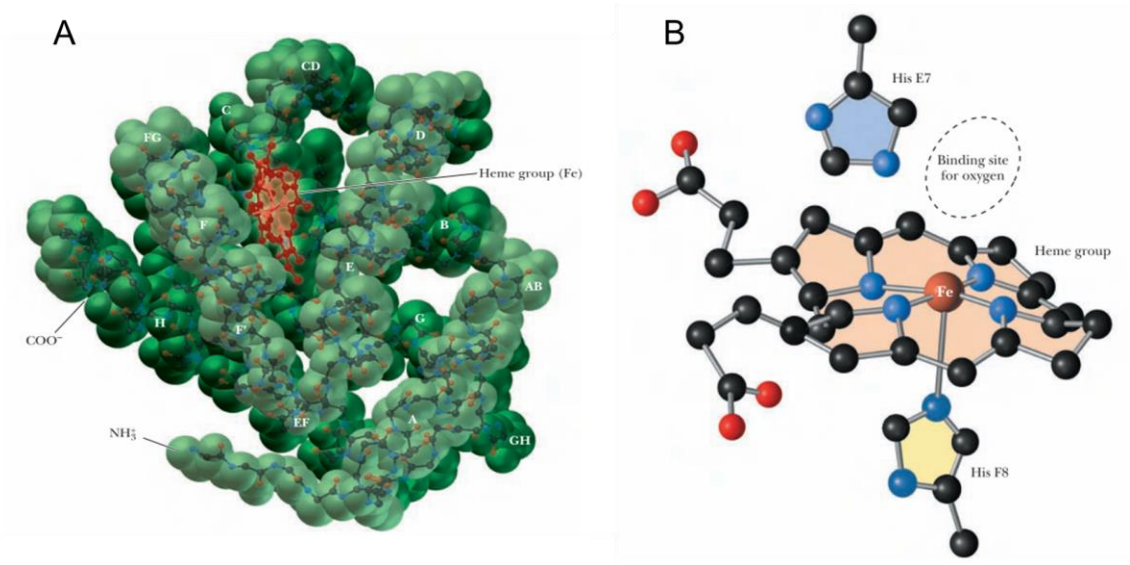


Figure 2.4. Structure of myoglobin and prosthetic heme group.

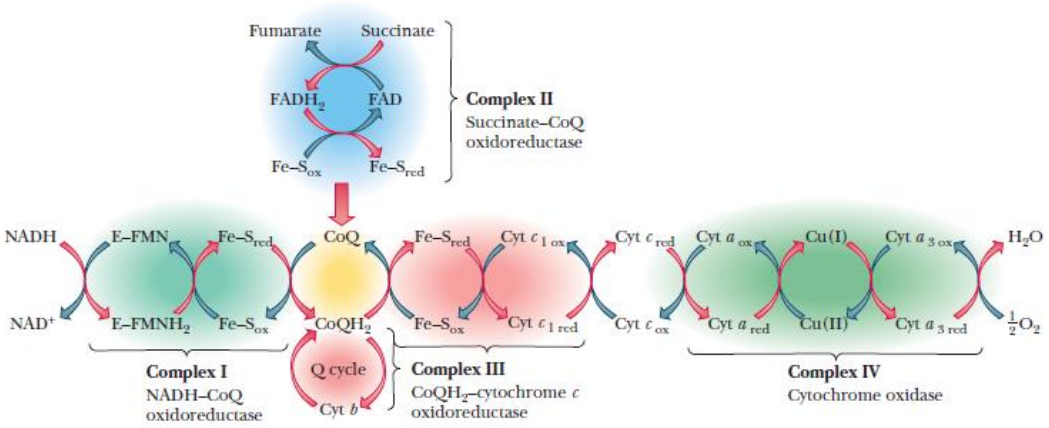
(A) Myoglobin is a structure that is used to store oxygen. It consists of a single polypeptide chain of 153 amino acid residues and the prosthetic heme group. Here you see a three-dimensional model of myoglobin showing the heme group (in red) and the α-helical segments where the amino acid residues are found. (B) The oxygen binding site of myoglobin. The porphyrin ring occupies four of the six coordinating sites of the iron. Proximal histidine occupies the fifth and oxygen is bound at the sixth site of the iron [23]. Image taken from M. K. Campbell and S. O. Farrell, 1976.

In general, the major use of iron in mammalian systems is that of oxygen transport by hemoglobin and oxygen storage by myoglobin [27]. However, many other cellular proteins require iron for their function. There are many other heme-containing proteins and enzymes, also called hemoproteins, the most common being cytochromes [27], [38]. They contain heme as their prosthetic group and their primary function in the cells of not only mammals, but all plants, animals and microorganisms, is electron transport [27], [38]. In cytochromes, the iron in the heme group does not bind to oxygen, but instead is involved in a series of redox reactions, reducing iron to Fe^{2+} and oxidizing it to Fe^{3+} [23], [27]. This is different than the iron in the heme group in hemoglobin, which remains in the reduced form as Fe^{2+} [23]. There are differences

in the side chains of the heme group of the cytochromes, but they all play an important role in different stages of the mitochondrial respiratory chain, as electron-transfer molecules [23], [27].

The electron transport chain (ETC) involves four multiunit membrane-bound complexes and two mobile electron carriers (coenzyme Q and cytochrome c). As previously stated, heme iron is key for the reactions to take place, as part of the cytochromes. In addition, nonheme iron proteins are also integral for the respiratory complexes within the ETC. The major group of nonheme iron proteins, are the iron-sulfur cluster (ISC) proteins (Figure 2.5), which are essential for mitochondrial respiration, the citric acid cycle, nucleotide metabolism, and a wide variety of other cellular functions [23], [27].

A



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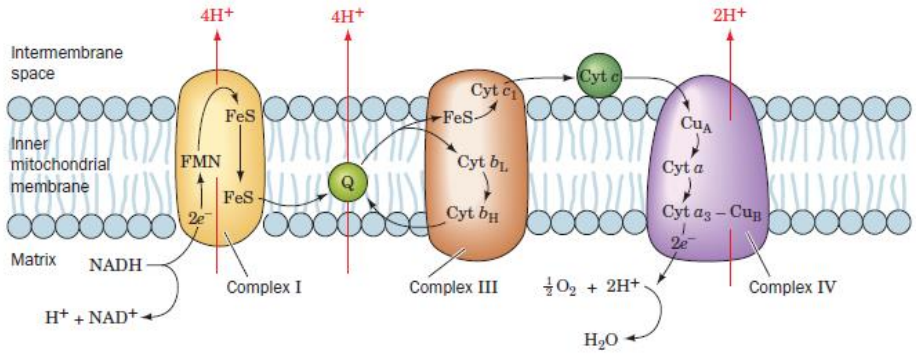

Figure 2.5. Respiratory complexes of the electron transport chain.

(A) The electron transport chain, outlining the respiratory complexes involving iron. An integral part of the complexes are the iron-sulfur clusters, in addition to iron being essential in the formation of cytochromes. (B) In the reduced cytochromes, the iron is in the Fe^{2+} state and in the oxidized cytochromes, the oxygen is in the Fe^{3+} oxidation sate. Image taken from M. K. Campbell and S. O. Farrell, 1976.

ISC have outstanding structural adaptability as they can exist in different configurations depending on the number of iron and sulfur atoms present [31], [39]. The common forms are rhomboid, cuboidal and cubane clusters, which are [2Fe-2S], [3Fe-4S], and [4Fe-4S] respectively [31], [39]. The iron in the Fe-S cluster can exist either as ferric or ferrous iron and cycle between the redox states, allowing them to participate in redox reactions [31]. The biosynthesis of the Fe-S clusters is regulated by the mitochondrial iron-sulfur cluster assembly machinery [31], [39], [40]. Despite the chemical simplicity of Fe-S clusters,

their biosynthesis is rather complicated and requires more than two dozen components in eukaryotes [31], [39], [40]. More importantly, the biosynthesis of these clusters plays a regulatory role in cellular iron metabolism. As an iron-consuming process directly controls the supply of iron, this makes sense biologically [38], [39]. This regulatory mechanism is remarkable, as heme synthesis does not directly affect the iron supply [38], [39]. Ultimately, iron plays an essential role in many vital metabolic processes, which in turn help regulate and control iron homeostasis.

2.2.4 Iron homeostasis

Iron uptake from nutrients, and its regulation and transfer, are tightly controlled and depends on specific cellular carrier mechanisms. The determinant of how much iron is absorbed is the systemic need for iron [26]. Iron absorption is balanced against iron loss through soughed intestinal mucosal cells, menstruation, and other blood losses [41]. Iron homeostasis is maintained entirely through the regulation of absorption and the recycling of iron, as it is highly conserved and reused by the body [41], [42]. Any disruption in the processes to maintain homeostasis can result in a variety of disorders associated with iron deficiency or overload.

The iron cycle in the body begins with dietary iron being taken up by the enterocytes of the upper gastrointestinal (GI) tract [9], [41], [43]. Small amounts of iron can be absorbed by the distal parts of the GI tract, but the proximal parts of the small intestine, specifically the duodenum and the first part of the jejunum, are predominantly used for absorption [9], [25], [26], [41]–[45]. Dietary ferric iron is reduced to the more soluble ferrous iron by cytochrome B at the apical border of the duodenal enterocytes and transported into the cell by a transporter called divalent metal transporter 1, or DMT1 [9], [25], [26], [41]–[45]. Once in the enterocyte, the ferrous iron can enter three different pathways depending on the requirements of the body. It can be taken into the mitochondria for heme synthesis, sequestered in the cell within the iron storage protein ferritin and shed into the gut lumen at the end of the enterocytes lifespan, or transferred to the basal transporter, ferroportin 1, for release into the body [41]–[45]. Recently absorbed iron or iron released from storage is distributed around the body in the circulation as transferrin [41]–[45].

A transferrin molecule can bind one or two atoms of ferric iron, which is mediated by receptors on the cell surface [41]–[45]. The iron can then either be stored as ferritin or used in the cell, such as for hemoglobin synthesis [41]–[45]. Storage of unused iron is crucial for iron homeostasis, as free iron is quite toxic. It enables iron to be sequestered in nontoxic form while providing a reservoir from which it can be used for future metabolic requirements [41]–[45].

The regulation of iron homeostasis is controlled at cellular and whole-body levels. If an individual requires more iron, more iron will be mobilized from iron stores in the body and there will be an increase in intestinal iron absorptions through an increase in transferrin receptors (TFR), such as TFR1 and TFR2 [41]–[44], [46]. Similarly, if the body iron is satisfied, these processes will be downregulated. One of the primary iron regulators is hepcidin, which is expressed in the liver [41]–[44], [46]. When systemic iron requirements are increased or iron stores are low, hepcidin production is decreased allowing more iron to enter circulation [41]–[44], [46]. When the body iron is high, hepcidin concentrations are high, and the iron supply to the plasma is reduced [41]–[44], [46]. Defective regulation of hepcidin or its receptor ferroportin causes a range of iron related disorders such as iron overload, caused by increased iron absorption, which can lead to excessive systemic iron accumulation and overload. Since iron is required for a number of diverse cellular processes, a constant balance is needed between uptake, transport, storage, and utilization to maintain homeostasis. Otherwise, serious health effects can occur.

2.3 Iron related diseases

Despite being an indispensable micronutrient for maintaining life, iron related health concerns can occur because of too much or too little iron. It is well known that iron has cytotoxic abilities, promoting the production of ROS. Hence, the levels of iron on a whole-body and cellular level are highly regulated. A breakdown of the iron maintenance processes leads to iron related diseases, which are primarily classified into two groups: iron deficient diseases and iron overload diseases.

2.3.1 Iron deficiency and anemia

One of the most common iron related diseases is iron deficiency (ID). It is the most widespread micronutrient deficiency in the world [27], [47]–[49]. Iron deficiency results from the depletion of iron stores which occurs faster than the amount of iron absorption, for an extended period of time [24], [47]–[49]. This could be due to the fact that there is a limited amount of biologically available iron is available to be ingested or excess blood loss caused by other pathologic infections or diseases [24], [47]–[49]. Nutritional iron deficiency is caused by the physiological requirements not being met by iron absorption [24], [47]–[49]. A common outcome of systemic iron deficiency, which is estimated to affect more then 2 billion people worldwide, is anemia [27], [47]–[49]. Anemia is characterized by a deficiency in the number of mature erythrocytes, which unavoidably lowers the oxygen carrying capacity of blood [27], [47]–[49].

Iron deficiency anemia (IDA), occurs secondary to iron deficiency and is one of the most common forms of nutrient deficiencies. It occurs when ID is severe enough to reduce erythropoiesis [27], [47]–[50]. IDA is clinically characterized by hypochromic anemia, meaning that the red blood cells have less hemoglobin than normal, and microcytic anemia, meaning that the red blood cells are also smaller than normal [27]. Similarly like ID, IDA represents a situation in which the iron requirements of the body are not being met, iron stores are significantly depleted and insufficient iron is available for physiological functions.

Some of the other causes of anemia include sickle cell anemia and thalassemia. Sickle cell anemia is one of the most common inherited diseases [24], [42], [51]. It is caused by a mutation in the gene that tells your body to make the iron-rich compound that makes blood red and enables red blood cells to carry oxygen from your lungs throughout your body, hemoglobin [23]. It is a disorder that affects the hemoglobin molecule, causing the cell to change shape when stressed [24], [42], [51]. The change in shape of the hemoglobin can cause problems with the red blood cell (RBC) structure and they become crescent shaped (sickled cells), making them rigid, sticky and misshapen [24], [42], [51]. When sickle cells move through small blood vessels, they can get stuck, blocking blood flow and causing pain which can result in serious health effects [23]. Additionally, another hemoglobin related cause of anemia is thalassemia, which is a

result from a genetic abnormality involving mutations of the genes responsible for hemoglobin production [24], [52]. This inherited condition effects the alpha and beta globin chains within hemoglobin, and an insufficient amount of normal hemoglobin type molecules are made [24], [52]. Again, as with any other hemoglobin disease, an insufficient amount of hemoglobin means not enough oxygen being delivered to all the cells in the body, resulting in mild to severe health concerns.

Anemic disorders, such as the ones previously mentioned, are often treated with iron supplements to replenish some of the depleted sources of iron stores caused by abnormal hemoglobin or iron deficiency [9]. The ease of availability of these iron supplements can cause overuse and lead to excess iron overload.

2.3.2 Iron overload

Iron related diseases stem from a disturbance in the homeostatic regulation systems in the body. Iron overload can be associated with a wide range of genetic and environmental factors, which can lead to serious health effects. The most widely known genetic iron overload disease is hereditary hemochromatosis (HH) [42], [53], [54]. HH is a disease that causes the body to absorb too much iron, leading to iron overload and iron accumulation in specific organs including the liver and heart [42], [53], [54]. Since humans cannot increase the amount of iron excreted, excess iron can overload and cause serious damage.

In addition to iron overload diseases, the use of iron supplements to treat iron deficient diseases can cause iron overload. Iron supplements are readily available and often overused in society. Overuse of iron supplements to treat iron anemia is one of the main causes of dietary, or secondary iron overload [55], [56]. Two main modes of iron overload include acute ingestion of a large amount of iron over a short period of time, and the second involves chronic daily ingestion of lower levels of iron over longer periods of time [55], [56].

Acute iron ingestion, is the ingestion of an overdose of pharmacological iron supplement, which is one of the most commonly reported situations leading to iron mediated toxicity [55], [56]. Iron overload is especially common in children ingesting multiple iron supplement tablets leading to overdose [9]. There are five phases of iron mediated toxicity due to acute iron overdose. Phase 1 is GI toxicity leading to

vomiting, 2 is dormancy, 3 cardiovascular shock and acidosis which is where death might occur, 4 liver toxicity, and 5 GI scarring and bowel obstruction, leading to stenosis [9], [57], [58]. The severity of symptoms depend on the amount of iron ingested. The toxicity of iron is surprisingly limited given that such large amounts are needed to observe death, but death by overdose is possible [9], [57], [58].

Chronic excess iron ingestion is much less common, and would occur from over ingestion of iron from normal diets; however it has not been reported [9], [59]. A common clinical occurrence of iron overload comes from patients that have haemoglobin defects such as sickle cell diseases. Many of these patients require monthly blood transfusions [59]. Given that RBCs are rich in iron and that iron is very efficiently recycled and not secreted, these patients become iron overloaded. Such chronic iron overload requires 10 years of so before iron becomes toxic [60], [61]. Microscopic examination of cardiac and liver cells suggests that vast excess iron is stored in large vacuoles in these cells and that seems to prevent short term toxicity [60]. Such vesicles can also be seen in mammalian cultured cells if they are fed excess iron for prolonged periods of time [60], [61]. Thus belies some of the complex mechanisms that organisms can use to prevent iron overload toxicity. On the other hand, it is not known why chronically elevated levels of iron can lead to the development of diseases including cancer, heart disease and other neurological diseases [9], [60], [61]. Overall, studies have shown that iron deficiency and excess iron overload are frequent and serious health concerns, which affect every cell and the organism as a whole.

2.4 Programmed cell death

Cell death is an essential element of many biological processes. It is a process that has essentially remained unchanged throughout evolution in multicellular organisms that is important for morphogenesis during development and for the maintenance of tissue homeostasis [62], [63]. One of the first necessary roles of cell death is during intrauterine embryonic development [62], [63]. Fingers and toes are sculpted by cell death, starting out as larger shapes and the individual digits separate only as the cells between them die [62], [63].

Cell death can be fundamentally classified into two basic forms, namely apoptosis or programmed cell death (PCD) and necrosis. The term PCD was introduced to define the process of programmed and controlled cellular self-destruction [62]–[64]. PCD plays an important role during development, predominantly in development, differentiation, proliferation, and epigenetic self-organization processes [62]–[64]. For organisms to function properly, PCD is crucial for the maintenance of tissue homeostasis, the removal of malfunctioning and harmful cells, and for defense against infections [62]–[65]. For example, virally infected cells induce cell death to self-destruct in a bid to destroy invading viruses and prevent the virus from spreading to other cells [63].

Activation of PCD occurs through genetically encoded processes that include signalling of biochemical pathways, resulting in the activation of cellular events that lead to controlled cell destruction [3]. The characterization of PCD occurs via processes that can be monitored cytologically and biochemically. These include processes such as cell shrinkage and swelling of organelles, mitochondrial outer membrane permeabilization, condensation and aggregation of the chromatin, nuclear fragmentation and plasma membrane blebbing (Figure 2.6) [62], [64].

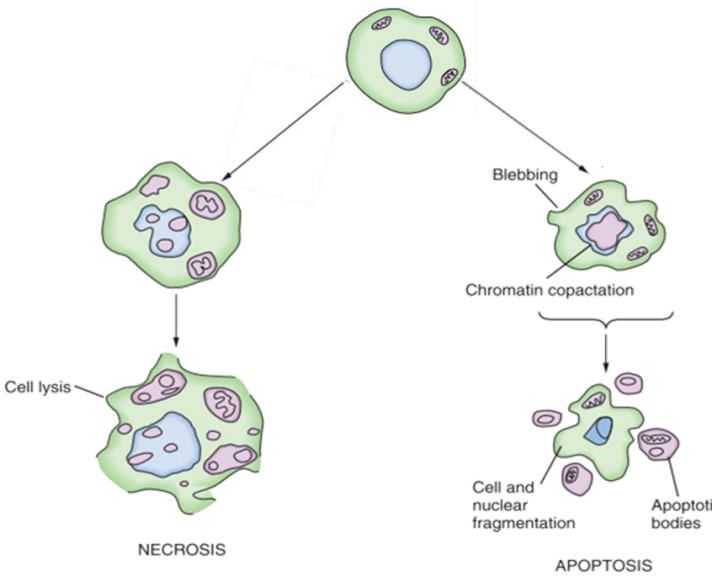

Figure 2.6. Typical cytological hallmarks of cell death.

A schematic diagram of morphologic changes that characterize cell death by necrosis and apoptosis. Necrosis culminates in cell lysis and provokes inflammation. Apoptosis cells are packaged into apoptotic bodies that are then engulfed by adjacent cells without an inflammatory response [62]. Image modified from S. R. D'Mello, 2007.

Although PCD is the most frequent form of regulated cell death, there are other types of cell death especially as necrosis. Necrosis describes uncontrolled or accidental cell death, which is a consequence of exposure to extreme stress. Stresses can include mechanical damage, exposure of cells to toxins and severe environmental perturbations [62], [64]. The cell death morphology during necrosis is distinguished by cellular swelling, dilation of organelles, mechanical rupture of the plasma membrane, and release of cellular content leading to an inflammatory response (Figure 2.6) [62], [64].

2.4.1 Stress activated programmed cell death

Within the body, there is an equilibrium between the net growth rate and the net rate of cell death. With exposure to cellular stress, the biological homeostasis is vulnerable and, depending on the type and severity of cellular stress, the cell's response can be manifold [63], [66]. Essentially, if the stress does not exceed a certain threshold, the cell can respond with an appropriate protective cellular response, to ensure

cell survival [66], [67]. On the other hand, if the cell fails to respond with a protective response, or if the stressful stimulus is too strong, the result will be activation of cell death pathways [66], [67].

Some of the cellular responses to stressful stimuli are the heat shock response, the unfolded protein response, the DNA damage response, and the response to oxidative stress, which is the most relevant response to our research [66]–[68]. ROS can originate from intracellular or extracellular sources. The most common oxidation reactions, which are involved in the electron transport chain, are required for mitochondrial respiration producing toxic free radical intermediates (Figure 2.1). As noted above, the production of free radical intermediates, which in the presence of iron, can produce highly reactive $OH^{\cdot}$ radical, via the Fenton reaction (Figure 2.2). All cells have free radial scavenging systems to minimize the oxidative damage from ROS, which requires appropriate proportions of molecular oxygen and various antioxidants [66], [67]. Normally in cells there is an equilibrium between pro-oxidant species and antioxidant defense mechanisms including ROS-metabolizing enzymes and compounds [66], [67]. When there is a disturbance in cellular redox balance, oxidative stress occurs [66]. Although most oxidative disruptions can be overcome by the cell's natural defenses, sustained agitation of this balance may result in either PCD or necrotic cell death [66], [67]. Of general interest here is that the use of antioxidant compounds such as vitamin C and E in our diets and other products like skin creams, are meant to counter stress mediated increases in ROS that occur during aging and other stresses such as viral infections [69]. Thus, Vitamins C and E are both able to absorb extra electrons without themselves becoming free radicals. This process is commonly observed every day as the Vitamin C in lemon juice can be used to prevent food oxidation processes like the air oxygen mediated browning of a cut apple [69].

Ultimately, the cellular stress response and adaptation mechanisms are numerous and highly complex. Using a well-studied model organism, such as the yeast *Saccharomyces Cerevisiae*, can help to enhance understanding of the complexity of PCD and cellular stress responses.

2.5 Yeast as a model to study mammalian PCD

Studying basic cellular processes, including PCD, is quite complex [67], [70]. A cells decision to die during PCD is regulated by a sophisticated network of genes and cellular processes [67], [70]. The complexity of these mechanisms in mammalian cells is such, as that they are difficult to study. Part of this is the fact that there is a great deal of redundancy and overlap in all the cell death and cell protective/ survival processes. Because yeast lives as a singe cell, it has become a very powerful research model in several areas of cell biology [70], [71]. Thus, yeast has been extensively used for investigating and understanding fundamental cellular and molecular processes [64], [70], [71]. This is why we use the yeast *S. cerevisiae*, which is the most extensively studied yeast, as a model organism to study the complexity of mammalian processes. Due to its methodological and logistic simplicity, *S. cerevisiae* makes an ideal model organism that is efficiently used to decipher the complex processes of higher level organisms [70], [71]. Yeast cells, which are eukaryotic cells, are straightforward to handle, control and manage. Some of the benefits of using yeast as a model organism include the fact that it grows quickly, in cheap defined media, and it can be grown in controlled conditions [64]. More importantly, the basic cellular processes that are observed in yeast are highly conserved in mammalian cells. Thus, the basic cellular processes in both yeast and mammalian cells have the same evolutionary origins and have maintained the same mechanisms [64]. This is evident from the fact that research, using yeast, has led to major discoveries about basic cellular processes and have been awarded Nobel prizes in medicine. The awarding of several Nobel prizes in physiology or Medicine to researchers using yeast clearly demonstrates that yeast has led to a number of breakthroughs in our understanding of processes that are basic to human biology [72].

In addition, many different studies with yeast have contributed to unravel some of the molecular mechanisms involved in the pathogenesis of many disorders, either through conventional complementation assays or by advancing humanized yeast systems [64]. For example, for several neurological disorders such as Huntington's disease, Parkinson's disease, and Alzheimer's disease, yeast studies contributed significant insights into the role of human proteins that are involved the disorders [64]. In addition to the experimental

studies in this field, recent advances in modeling of PCD in mammalian cells have provided us with a network that proposes interconnectivity within and between different PCD pathways [64].

2.5.1 Yeast to study iron mediated PCD

Yeast PCD has been observed to occur in response to the same stimuli that induce mammalian PCD, which include oxidative stress, exposure to acetic acid, and the expression of mammalian pro-apoptotic proteins [71], [73]. Yeast cells display numerous characteristics of apoptosis, including chromatin condensation, DNA breakage, accumulation of ROS, and release of pro-death factors from mitochondria [71], [73]. Therefore, yeast contains a fundamental PCD process that shares many processes with mammalian cells, making it a major contributor to the study of human diseases [71], [73].

Of importance to this research is the fact that the metabolism of most micronutrients is essentially the same for yeast cells as it is for mammalian cells. This is of note for elements like iron and copper, where yeast is used as a model to understand iron metabolism [9]. The Greenwood lab has made extensive use of yeast as a model to study PCD. Most of the studies have focused on the study of humanized yeast. The lab have identified and characterized human genes that can prevent PCD in response to stresses when expressed in yeast [3]. More recently, research has shown that iron mediated PCD in yeast differs from the PCD that is induced by other similar compounds like copper [3]. In these studies, it was found that iron, even at sub-lethal concentrations, led to the formation of a precipitate with yeast media that is of unknown origin. For this thesis, it is proposed that exogenous iron causes precipitation that precedes iron mediated cell death in yeast, and is responsible for the ability of iron to induce cell death in yeast. A series of experiments have been carried out that support this hypothesis.

Chapter 3 – Manuscript

Iron mediated cell death involves the precipitation and depletion of iron-phosphate.

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3.1 Abstract

Iron is an essential micronutrient and since it interconverts between ferrous (Fe^{+2}) and ferric (Fe^{+3}) oxidation states, it is very useful in electron transfer reactions and oxidative metabolism. In excess, iron is thought to be toxic by entering cells and generating Reactive Oxygen Species that can serve to induce Programmed Cell Death (PCD) and even necrosis. Studies of iron toxicity are limited by the fact that iron precipitates in complex salt solutions like cell growth media containing high levels of phosphate. Using agar based growth assays we show here that wild type yeast can grow unaffected in the presence of ferric chloride at 5mM. In effect growth inhibition was only noticeable at 7mM, which represents a 6000 fold increase in the basal concentration of iron present in YNB yeast media. Addition of ferric chloride to YNB yeast culture media alone resulted in the dose dependent accumulation of precipitate matter. A maximum precipitate of 4.5g/L of YNB media was detected with 20mM ferric chloride. The accumulation of precipitation was seen at doses as low as 1mM. Half-maximum accumulation of precipitate was detectable with the lethal 8mM dose of iron. Stoichiometric analysis combined with the solubility of iron with the different salts in YNB suggests that the precipitate corresponds to ferric phosphate. Overall, these results indicate that excess extracellular iron is toxic due to phosphate depletion. These results are consistent with numerous other studies linking iron and phosphate insolubility to nutrient depletion mediated growth impairments and cell death.

Keywords: programmed cell death; iron mediated cell death; copper mediated cell death; iron solubility; iron phosphate.

3.2 Introduction

Intracellular space differs significantly from the extracellular space in which the cell resides [74]–[77]. Maintaining this distinction is primordial for the existence of life. The cell has evolved a large variety of processes to maintain control over the content of its interior. Of importance is the semi-permeable lipid bilayer that surrounds the cell [74], [75]. This serves as a physical and chemical barrier that prevents entry to unwanted compounds. The cell is further equipped with a variety of different highly specific transport proteins that are present in the lipid bilayer that promote the entry of chemical compounds and substances that are required for normal cellular processes. Not only is the cell highly selective in what it permits to enter the cell, it is also very quantitative in the amount of different compounds that it lets enter [75], [78]. For example, certain essential nutrients like glucose are required at higher concentrations than essential micronutrients like iron [78], [79]. Thus, there are a variety of mechanisms involved in ensuring the required cellular milieu is maintained. These regulatory processes show complexity and adaptability. For example, chronic exposure to an excess of many different chemicals activates processes that prevents excess over-accumulation [78]. A prime example are the complex strategies, including decreasing influx and increasing efflux, that cells invoke to acquire resistance to different chemotherapeutics [80].

These homeostatic regulatory mechanisms are as expected, readily apparent in single celled organisms given that they must live in often extreme, chaotic and chemically variable environments [81], [82]. On the other hand, these processes are also critically important in more complex mammalian cells in spite of the fact that their extracellular environment is largely controlled by internal processes including the central nervous system (CNS) [78], [83]. Thus cellular homeostasis maintains life by assuring that the conditions on the inside of the cell are kept within a very narrow range for many different parameters no matter what the extracellular condition [84], [85]. When extracellular conditions change and become unfavourable, the cell adapts all the while maintaining complete control over its intracellular milieu. If the conditions become more extreme, the cell will trigger cellular suicide as a way of maintaining control under conditions that may lead to cellular damage [86]. They will lose control only if the conditions become so unfavourable to life so as to actually destroy the cell in a process that is normally called necrosis [75]. In

effect, extracellular changes do not usually lead directly to changes in the intracellular milieu, instead extracellular changes are largely sensed by activation of second messenger systems [76]. For example, compounds like adrenaline mediate dramatic cellular changes without entering the cell. Instead, adrenaline binds to extracellular receptors that mediate their effect by activating adenylyl cyclase which then increase the second messenger cAMP. Similarly, mechanisms exist for the cell to sense the extracellular and the intracellular levels of micronutrients like iron and phosphate and to vary their rates of uptake accordingly [78], [79]. Nevertheless, clearly some compounds can bypass the regulatory process and can thus accumulate in a cell. For example, many compounds like ethanol can enter the lipid bilayer, accumulate within the cell, and cause stress that can lead to PCD and even necrosis. Although it is known that most chemicals can lead to cell death if given at sufficiently high doses, most normal chemicals still do not enter the cell even at extreme concentrations [75], [78], [87]. In spite of the fact that certain intracellular signals such as ROS increase in response to most stresses, it remains largely unknown how most extracellular stresses are transduced into cellular responses [76], [88].

As mentioned, there are a large variety of extracellular compounds that are required inside the cell [78], [81]. The mechanisms by which many of these compounds enter cells and how they are maintained at appropriate concentrations has only been studied for a small number of compounds; especially essential macronutrients like glucose and essential micronutrients like iron and phosphate [78], [79], [89]. Iron is of interest since it is critically important for the function of a large variety of different enzymes [9], [79]. Further, since iron can interconvert between ferrous (Fe^{2+}) and ferric (Fe^{3+}) iron oxidation states it very useful in electron transfer reactions and thus it plays key roles in processes like the mitochondrial electron transport chain and oxidative metabolism. This interconversion of oxidative states also means that iron can participate in inappropriate oxidative/reductive reactions (Fenton and Haber-Weiss reactions) that can lead to the productions of ROS. Thus excess intracellular iron is thought to be stressful leading to apoptotic responses or even to necrosis [9], [54]. If potential toxicity were not enough, there are a number of other obstacles in the use of iron for biological systems. Therefore, even though ferrous iron is readily soluble, it easily gets interconverted to the far less soluble ferric iron oxidative state. Thus although iron is plentiful,

the more biologically available ferrous form is often limited. Thus, it is not surprising that iron is zealously guarded and rarely discarded. For example, in mammals large amounts of iron containing hemoglobin is continuously degraded as erythrocytes are short lived. In spite of this, iron is efficiently recycled and reused with very little loss [9], [90]. Finally, water soluble ferric iron forms strong ionic bonds with a large variety of compounds including phosphate and carbonate anions [91]–[94]. In addition to being converted to its more soluble ferrous state by the action of oxygen, ferric iron will also commonly precipitate when added to complex solutions like cell or plant culture media [91], [95], [96]. Thus, transferrin or soluble chelators of iron such as EDTA and citrate, are commonly used to keep iron in solution even though chelated iron is less biologically available and far less toxic [9], [91], [94], [95], [97].

Although lack of adequate iron is more common, there are nevertheless quite a few clinical conditions where patients are faced with chronically high levels of iron [80], [82]. As a consequence, numerous mammalian cell culture systems as well as model organisms, including yeast, have been used to study iron toxicity [3], [77], [80], [81], [98]–[101]. Many of these studies suggests that excess extracellular iron enters the cell and is toxic due to its ability to overcome cellular defences and cause damage by the production of ROS [54], [90], [100]. This conclusion appears to conflict with basic concepts of homeostasis and with the studies showing that very little excess iron actually enters cells [3], [79], [87]. It is of further interest that of all the existing studies, we were unable to find any published reports using iron alone to induce cell death [9], [75]. Instead, a second stress is always used in order to observe iron mediated adverse effects. The additional stress is commonly serum withdrawal for mammalian cells or the introduction of a mutation that renders cells more sensitive to iron stress for yeast [3], [86], [100], [102]. The second stress is likely required to decrease the precipitation of the excess iron with iron insoluble salts present in all growth media [91], [95], [103]. In effect, iron solubility has been long recognized in some fields but a largely under reported problem for the study of excess iron toxicity [91], [104]. Here, we extend the previous studies on iron toxicity and show that the addition of excess iron leads to the formation of precipitate at concentrations far lower than the concentration of iron required to induce cell death [3], [101].

Thus, it is proposed that iron-mediated cell death is not directly due to the toxicity of iron on its own; instead, iron toxicity is due to depletion and subsequent starvation of essential nutrients.

3.3 Materials and methods

3.3.1 Yeast strains and media

The yeast used in this study was S. cerevisiae strain BY4742. The yeast media used consisted of yeast nitrogen base (YNB) defined media, with 2% glucose and the addition of the required amino acids (leucine, lysine, and histidine) [105]. The plasmids used were the empty vector p426GAL1 as well as p426GAL1-14-3-3, which expresses the human 14-3-3β/α gene under the galactose inducible GAL1 promoter. Thus, 2% galactose and 1% raffinose were used instead of 2% glucose to induce the expression of 14-3-3 [106].

3.3.2 Spot assays

Spot growth assays were used to investigate toxicity of a chemical stress by observing the amount of growth inhibition [107]. BY4242 cells were first grown in liquid YNB glucose media, and then the saturated cultures were diluted 1 to 5 into fresh YNB galactose media. Next, the cells were incubated for 4 hours at 30ºC with agitation, serially diluted tenfold with sterile water, then aliquots of the dilutions were spotted onto nutrient agar media. The cells were then incubated for 96 hours at 30ºC. The nutrient agar plates were prepared beforehand with the desired concentrations of iron or copper stress. A minimum of three different spot assays were performed for each experiment shown and comparable results obtained.

3.3.3 Solubility and precipitation assays

Solubility and iron precipitation assays were used to determine the effect of increasing iron and copper concentrations, and the accumulation of precipitate in yeast media. The solubility assays started with 2mL of liquid YNB galactose media in multiple tubes and increasing amounts of iron or copper. The YNB galactose media without any additions was used as the control. The solutions were incubated at room

temperature for 24 hours, vortexed to ensure homogeneity, and then a photograph was taken. The precipitation assay was used to quantify the amount of precipitation. The precipitate that formed was collected by centrifugation, dried, and weighed each day until the weight no longer changed signifying any remaining liquid was evaporated. The data from the precipitation assay experiments are presented as the mean ± standard deviations of triplicate experiments repeated a minimum of three independent times. Statistical significance of the data was determined using analysis of variance (ANOVA) and Dunnett's Test, a post hoc statistical analysis. DMEM containing 10% FBS was used as the mammalian cell culture media [108]. Increasing amounts of iron were added to the media and the precipitate was visualized and quantified as described above for the yeast YNB media.

3.4 Results

3.4.1 Iron is a dose-dependent inhibitor of yeast growth

Iron is both an essential micronutrient as well as a well known cytotoxicant [54]. Its ability to gain and lose electrons as it interconverts between ferrous (Fe^{2+}) and ferric (Fe^{3+}) oxidation states makes it essential as an electron carrier in cellular processes [9], [54]. Conversely, excess iron is believed to be toxic for the same reason, since the interconversion of iron leads to free electrons and the production of ROS and the induction of cell death [9], [75]. Given that the intracellular levels of iron are tightly regulated even when cells are grown with large excesses of iron, alternative methods of iron toxicity must exist [3], [9], [87]. As a first step to investigate iron toxicity, the level of iron required to inhibit growth of yeast cells was determined using the spot growth assay [109], [110] (Figure 3.1). Freshly grown BY4742 yeast cultures, with the empty vector alone or with the 14-3-3β/α expressing vector, were diluted in fresh galactose YNB media and grown for 4 hours, in order to study growing cells. The cultures were serially diluted and 5µL aliquots of the dilutions were spotted onto nutrient agar media plates without and with increasing concentrations of iron as ferric chloride ($FeCl_3$). After spotting, the plates were incubated and allowed to grow. Growth of the yeast harbouring the empty vector p426GAL1 was largely unaffected by the addition

of 2mM and 5mM $FeCl_3$ (Figure 3.1A). At 7mM, there was noticeable growth inhibition, while at 8mM, there was no detectable growth on the plate. In these experiments, the yeast strain expressing the human 14-3-3β/α sequence was also used. These cells serve as a control because they are known to be supersensitive to iron [3]. As shown in Figure 3.1A, cells expressing 14-3-3β/α show growth inhibitions at lower levels of iron than the empty vector control. Therefore, it is confirmed that iron is a dose-dependent inhibitor of yeast growth. This is consistent with the widely reported toxicity of exogenous iron [9], [54]. What is not expected is the amount of iron required to show negative effects on yeast growth. Yeast YNB media is reported to contain 1.2μM ferric chloride [103]. The addition of 7mM iron thus represents close to a 6000-fold increase in the basal level of iron required for normal growth. Given the magnitude of iron required for toxicity, the question is, can iron be really considered as toxic [111], [112]?

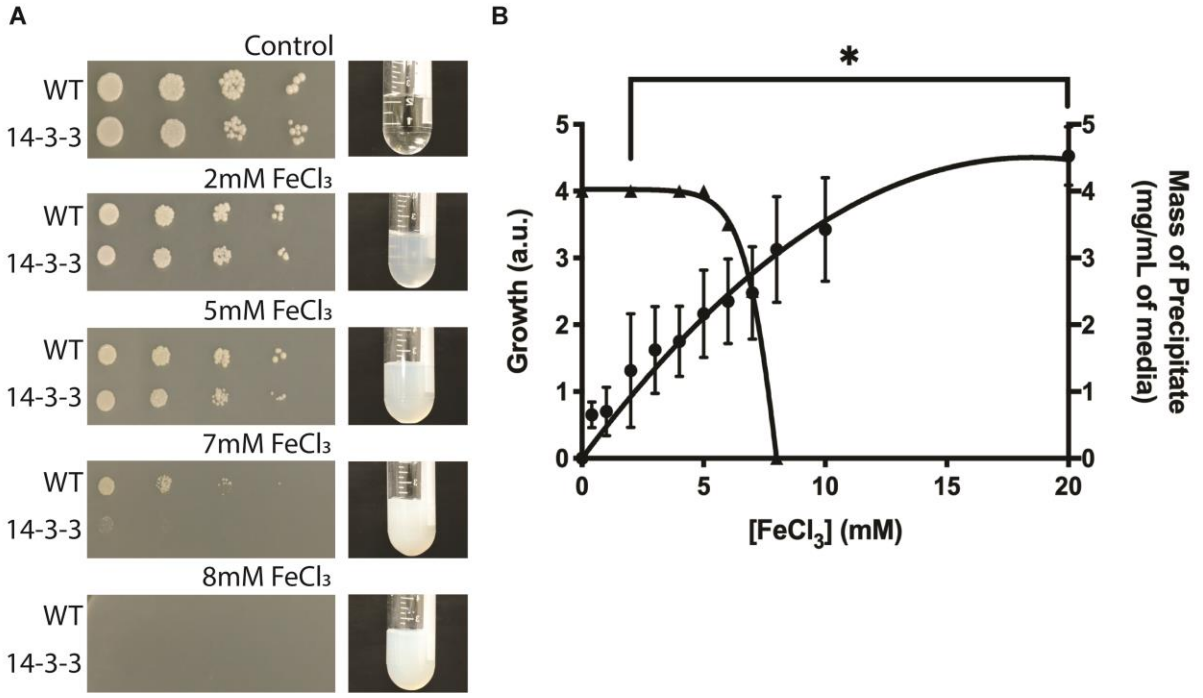

Figure 3.1. Iron is a dose-dependent inhibitor of yeast growth.

The effect of iron on yeast growth and the formation of a precipitate were analyzed. (A) Spot assays were used to assess the effect of increasing amounts of iron on yeast on wild type (WT) cells harbouring the empty vector p426GAL1 as well as cells harbouring the same vector containing a galactose inducible cDNA for the human 14-3-3β/α gene. Aliquots of freshly grown culture of both strains were serially diluted and aliquots were spotted onto nutrient agar plates containing the indicated concentrations of $FeCl_3$. The plates were incubated for 96 hours to allow the cells to grow and photographs of the resultant plates are shown. Additionally, the solubility of iron was examined by adding the indicated concentrations of $FeCl_3$ to 2ml of YNB Gal yeast media. The solutions were incubated for 24 hours, vortexed and photographs of the resultant tubes are shown. (B) Graphical comparison of the effects of $FeCl_3$ on yeast growth and formation of precipitation in YNB Gal/Raf +++ yeast media. The growth of yeast on YNB nutrient agar plates containing increasing concentrations of $FeCl_3$ was estimated semi-quantitatively (▲). The precipitation formed by the addition of different concentration of FeCl3 was collected by centrifugation and its weight was determined (●). These values are zeroed to remove the baseline precipitate/ residue naturally occurring when YNB media is centrifuged and the remaining water removed.*; indicates significant statistical difference between control WT cells and cells treated with iron ($p < 0.001$) using ANOVA and Dunnett's Test.

In a previous study, the addition of iron, at a concentration as low as 0.4mM, to yeast growth media lead to an accumulation of some unknown precipitate in the media [3]. The prevailing hypothesis was that iron was reacting with some component within the media and producing a precipitate, thus potentially contributing to iron mediated cell death. To explore this possibility, we determined the effect of

increasing iron concentration on the accumulation of precipitate in yeast media. YNB yeast media alone is a clear solution as shown (Figure 3.1A). In contrast, the addition of iron to the media leads to the formation of precipitate that was shown to increase with the addition of increasing amounts of iron.

To examine if the precipitation may be related to the effects of iron on yeast growth, a semi-quantitative dose-dependent precipitation experiment was performed. The YNB media with no additions was used as the control. After the addition of the different concentrations of iron, the solutions were incubated for 24 hours and photographed to qualitatively document the precipitation (Figure 3.1A). At concentrations of $FeCl_3$ from 2mM to 5mM, it was observed that there was not only precipitate forming; there was no associated inhibition of growth.

As previously observed, iron mediated cell death is also dose-dependent (Figure 3.1A). To determine the relationship between iron mediated cell death and iron precipitation, we quantitatively compared results from the spot assays and the solubility experiment. As seen in Figure 3.1B, both the amount of growth inhibition and the accumulation of precipitate are unique and distinct. The growth curve shows an initial phase up to 5mM $FeCl_3$, where there is normal growth and no visible growth inhibition. Subsequently, there is a precipitous decrease in the viability at 7mM, signifying significant growth inhibition and finally at 8mM complete inhibition is observed. In contrast, the precipitation curve was the opposite; as the concentration of $FeCl_3$ increased, the amount of precipitate accumulating also increased. The control with no addition of iron had no detectable precipitation. With increasing amounts of iron added from 0.4mM to 20mM, it was observed that there was there was a corresponding increase in accumulation of a precipitate (Figure 3.1B). Half-maximal accumulation was observed to occur at 5mM $FeCl_3$. The accumulation of precipitate began to slowly plateau at a maximum of 4.5mg±0.3. Overall, the mechanism of both processes supports the notion that the formation and accumulation of a precipitate may be responsible for the toxic effects of excess iron. This phenomena of nutrient depletion has yet to be described for yeast and mammalian cell culture media but is commonly documented for plant and plant cell culture [91], [113].

3.4.2 The oxidation state of iron has no effect on its toxicity

Iron exists in a wide range of oxidation states and can stably interconvert between ferrous (Fe^{2+}) and ferric (Fe^{3+}) forms [88]. These have different solubilities and differential biological functions as electron acceptor or donor. Cells are able to interconvert both forms of iron using ferroxidases. It is nevertheless of interest to determine if both the ferric and ferrous forms show similar effects on growth due to solubility. Spot growth assays were thus used here to determine if ferrous iron served to inhibit growth. Freshly grown yeast cultures were diluted and aliquots of the dilutions were spotted onto nutrient agar media plates without and with increasing concentrations of iron as ferrous sulphate ($FeSO_4$). After spotting, the plates were incubated for 96 hours to allow growth of the cells. The empty vector control cells grew normally at concentrations of 1mM and 10mM $FeSO_4$ (Figure 3.2). At 50mM, there was a noticeable decrease in growth, while at 100mM, there is no visible growth on the plate. As a positive control, we also show that the cells expressing 14-3-3β/α are supersensitive to the effects of added iron (Figure 3.2). Therefore, these results support the concept that ferrous iron, like ferric iron, is a dose-dependent inhibitor of yeast growth.

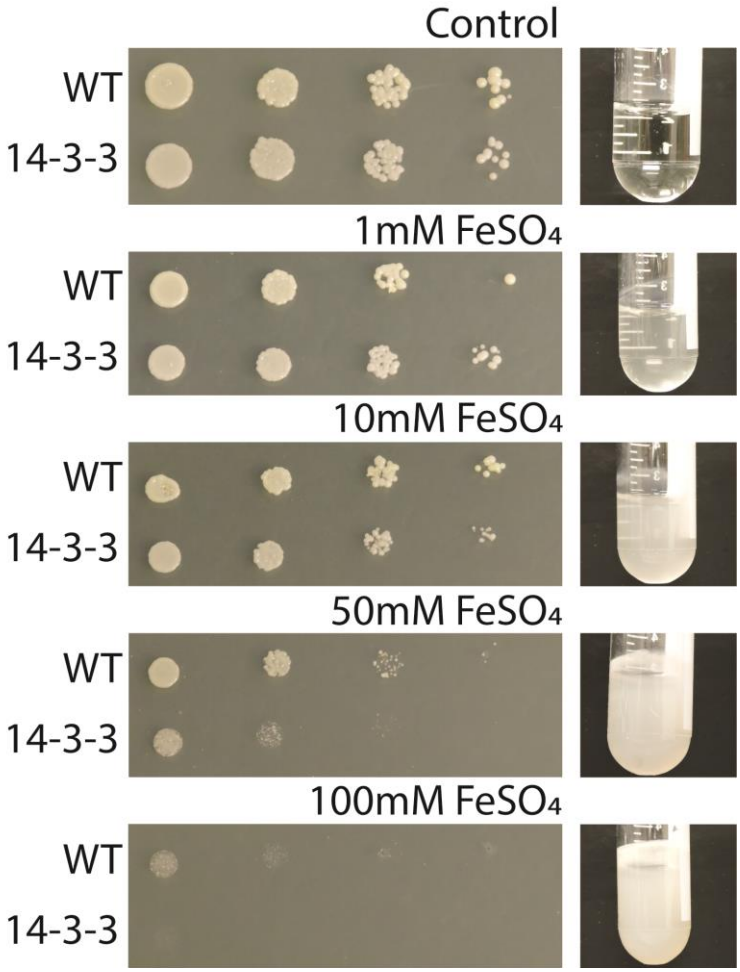

Figure 3.2. The oxidation state of iron does not affect its ability to inhibit growth.

The effect of ferrous iron on yeast growth and the formation of a precipitate was analyzed. Spot growth assays were used to determine the effect of increasing iron concentration on yeast wild type (WT) cells harbouring the empty vector p426GAL1 as well as cells harbouring the same vector containing a galactose inducible cDNA for the human 14-3-3β/α gene. Freshly grown cultures of both strains were serially diluted and aliquots were spotted onto nutrient agar plates containing the indicated concentrations of $FeSO_4$. The plates were incubated for 96 hours to allow the cells to grow and photographs of the resultant plates are shown. Additionally, the solubility of $FeSO_4$ was examined by adding the indicated concentrations to 2ml of YNB Gal/Raf +++ yeast media. The solutions were incubated for 24 hours, vortexed and photographs of the resultant tubes are shown.

As mentioned above, cells can utilize both forms of iron. Despite the differences in solubility, both forms are also capable of inhibiting yeast growth when given in excess. Nevertheless, it takes significantly more ferrous (100mM) than ferric (8mM) to prevent the growth of yeast (Figure 3.2 vs. Figure 3.1). It was of interest to determine if the ferrous form of iron also caused the formation of precipitate upon

its addition to yeast growth media. Increasing amount of $FeSO_4$ was added to YNB media and the resultant turbidity of the media is shown (Figure 3.2). The tube containing media alone with no added iron remained clear. Iron mediated precipitation was observed even as at the lowest concentration of 1mM $FeSO_4$. The precipitate formation was dose dependant with the opacity of the solutions increasing with increasing concentration of added iron (Figure 3.2). Thus even though it took more ferrous iron than ferric iron, both cause the formation of a precipitate in a dose dependent manner. Comparison of the growth inhibition and precipitation data demonstrates that ferric iron mediated growth inhibition occurs at a concentration of iron that is between 10mM and 50mM while precipitation is observed at concentration as low as 1mM. Thus ferric iron is like ferrous iron, in that precipitation occurs at concentration lower than is needed for growth inhibition (Figure 3.2).

3.4.3 Copper growth inhibition does not require precipitation

There are a number of transition metals that are essential micronutrients for biological systems, including iron, copper and zinc [79]. Similar to iron, excess copper can also be toxic to cells and can also lead to programmed cell death in mammalian as well as yeast cells [105], [114]. Given its similarity to iron, we wanted to determine if copper toxicity, like iron toxicity, is linked to the formation of copper induced precipitation in yeast growth media. To assess the effects of yeast cell growth of cells challenged with copper, spot growth assays were used. Freshly grown yeast cultures were serially diluted and spotted onto nutrient agar media plates without and with increasing concentrations of copper as copper sulphate (CuSO4). After spotting, the plates were incubated for 96 hours to allow growth of the cells (Figure 3.3). The yeast cells grown with 0.5mM or 1mM $CuSO_4$ showed similar growth as control cells grown without the addition of the copper (Figure 3.3). At 2mM, there was noticeable effect on growth, while at 50mM there was complete growth inhibition (Figure 3.3). As a control, we used the yeast cells harbouring the vector expressing human 14-3-3. As expected, these cells show increased resistance to copper as indicated by their ability to grow better than the wild type cells at 2mM copper (Figure 3.3) [3]. Therefore, copper, like iron, is a dose-dependent inhibitor of yeast growth.

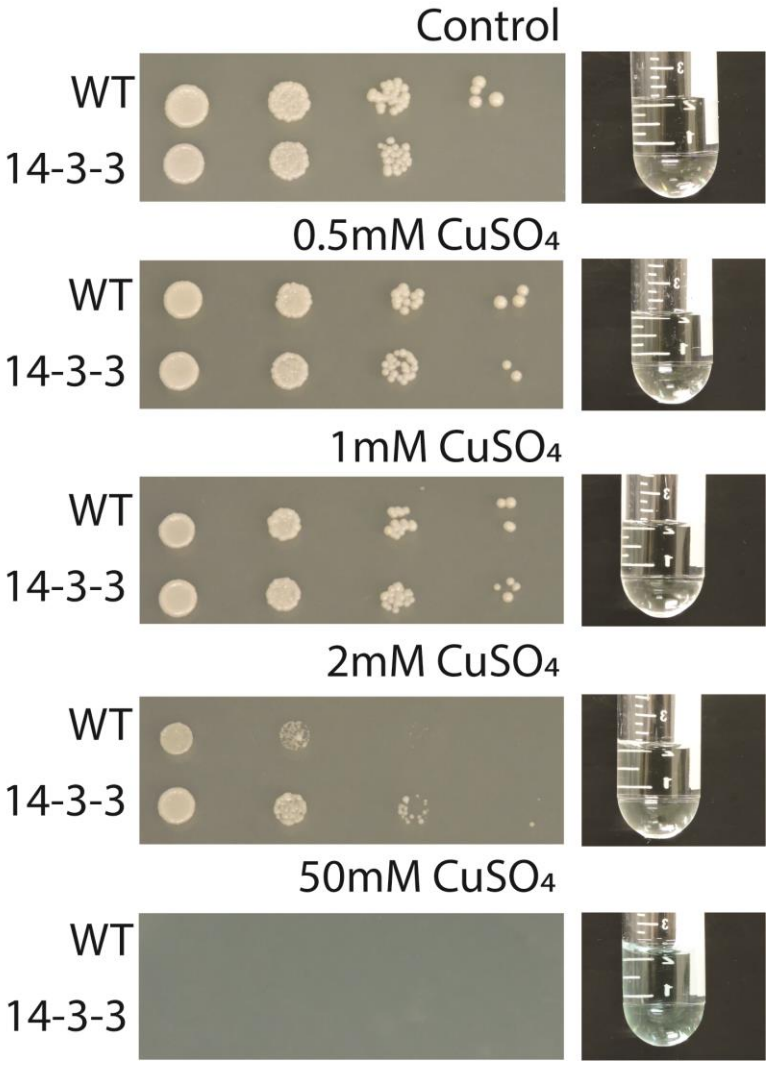

Figure 3.3. Copper growth inhibition does not require precipitation.

The effect of copper stress on yeast growth and the formation of a precipitate was analyzed. Spot growth assays were used to determine the effect of increasing copper concentration on yeast wild type (WT) cells harbouring the empty vector p426GAL1 as well as cells harbouring the same vector containing a galactose inducible cDNA for the human 14-3-3β/α gene. Freshly grown cultures of both strains were serially diluted and aliquots were spotted onto nutrient agar plates containing the indicated concentrations of $CuSO_4$. The plates were incubated for 96 hours to allow the cells to grow and photographs of the resultant plates are shown. Additionally, the solubility of $CuSO_4$ was examined by adding the indicated concentrations to 2ml of YNB Gal/Raf +++ yeast media. The solutions were incubated for 24 hours, vortexed and photographs of the resultant tubes are shown.

To determine if precipitation occurs with excess copper, increasing amounts of $CuSO_4$ was added to YNB media and the tubes were photographed after 24 h of incubation (Figure 3.3). The tube containing media alone contained no visible precipitation. Similarly, no precipitation was observed with concentrations of 0.5mM to 50mM $CuSO_4$ (Figure 3.3). These results suggest that unlike iron where the opacity of the solutions increased with increasing concentration of iron, copper remained soluble with no observable precipitation. Thus, precipitation of a component of the yeast media is not necessary for the toxicity of all transition metals.

3.4.4 Exogenous iron precipitates in mammalian cell culture growth media

The formation of a precipitate due to iron in yeast growth media prompted the question of whether this occurred in the growth media used for other types of cells. This is likely since the formation of precipitate in mammalian growth media is a recognized but under-reported problem when working with iron [9], [91], [95], [115]. To examine this, increasing concentrations of ferric iron was added to tubes containing DMEM media and photographs were taken after 24 hours (Figure 3.4A). The addition of iron to DMEM media as low as 1mM led to the formation of a precipitate (Figure 3.4B) Thus, excess iron also causes precipitation of a component of mammalian cell culture growth media [9].

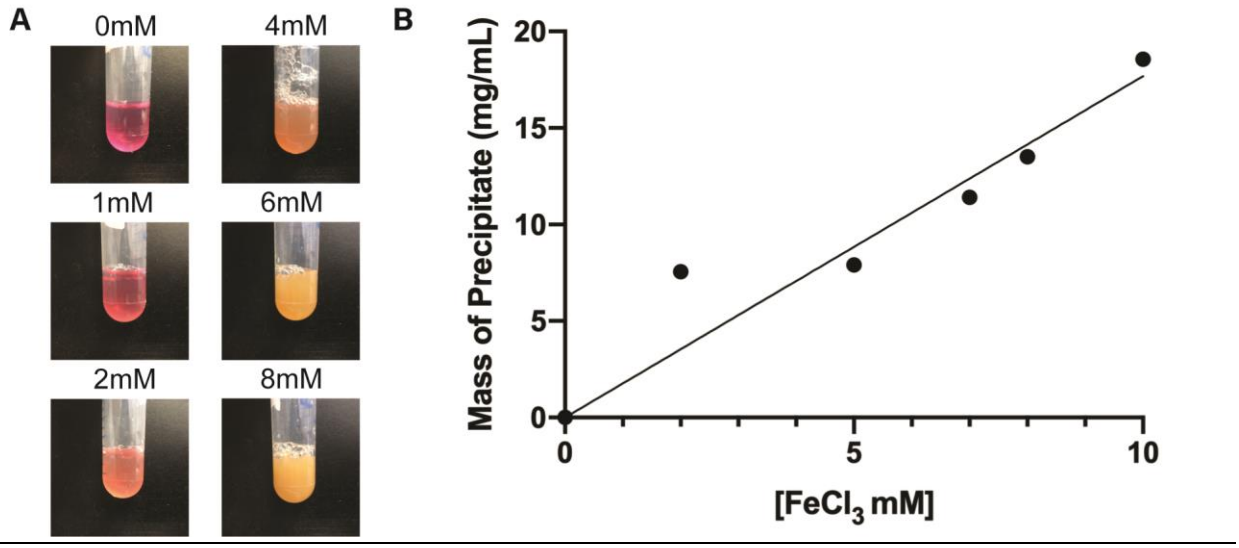

Figure 3.4. Iron precipitates in DMEM media with 10% FBS in a dose dependent manner.

A) The mass of precipitate formed is plotted as a function of ferric iron concentration in DMEM media. B) Qualitative images of aliquots of DMEM media supplemented with increasing concentrations of $FeCl_3$.

3.5 Discussion

Iron is an essential micronutrient that is required as a co-factor for numerous enzymes, as a functional part of the oxygen carrying heme required for hemoglobin as well as electron carrier in mitochondrial respiration [9], [54]. Its ability to accept and donate an electron as it interconverts between the ferric and ferrous oxidation states makes it well suited for its biological functions. This later feature also renders it potentially cytotoxic as iron can serve to generate free electrons and thus contribute to the production of Reactive Oxygen Species (ROS). High levels of ROS can serve as second messengers that may activate cellular processes such as apoptosis or if at sufficiently high levels, ROS may also directly damage cellular components and lead to uncontrolled forms of cell death as necrosis [76], [88]. Although the ferric form of iron is commonly reported to be very toxic, here we show that yeast can grow in the presence of excess iron even as high as 5mM (Figure 3.1A). Given that yeast minimal media YNB contains 1.2×10^{-6} M (200 μg/L as ferric chloride) iron (Figure 3.5), our results thus indicate that a 4000 fold excess

of iron has no toxic effects on yeast cells (Figure 3.1A) [3]. This reflects the fact that yeast, like all cells, have a large repertoire of cellular processes as well as a large repertoire of proteins that function to ensure that an adequate supply of iron is maintained all the while preventing potentially toxic excess iron from entering and disrupting the cell [9], [75], [79]. These include iron transporters whose levels vary in response to iron concentrations inside and outside the cell as well as intracellular proteins that prevent excess iron accumulation. Thus the intracellular concentration of iron is maintained within a narrow range in spite of large increases in the concentration of extracellular iron [9], [87]. For example, we have previously reported that yeast cells growing with a 10-fold excess of iron have modest 50% increases in their intracellular iron content [3]. This is the basic concept of cellular homeostasis. Cellular homeostasis is often overlooked especially in the numerous studies that assume that added extracellular micronutrients like iron just passively enter and equilibrate within the cell at concentrations sufficient to lead to cell death [78], [85]. In effect, a basic tenet of cell physiology is the maintenance of homeostasis when faced with changing extracellular environments [75], [84], [85].

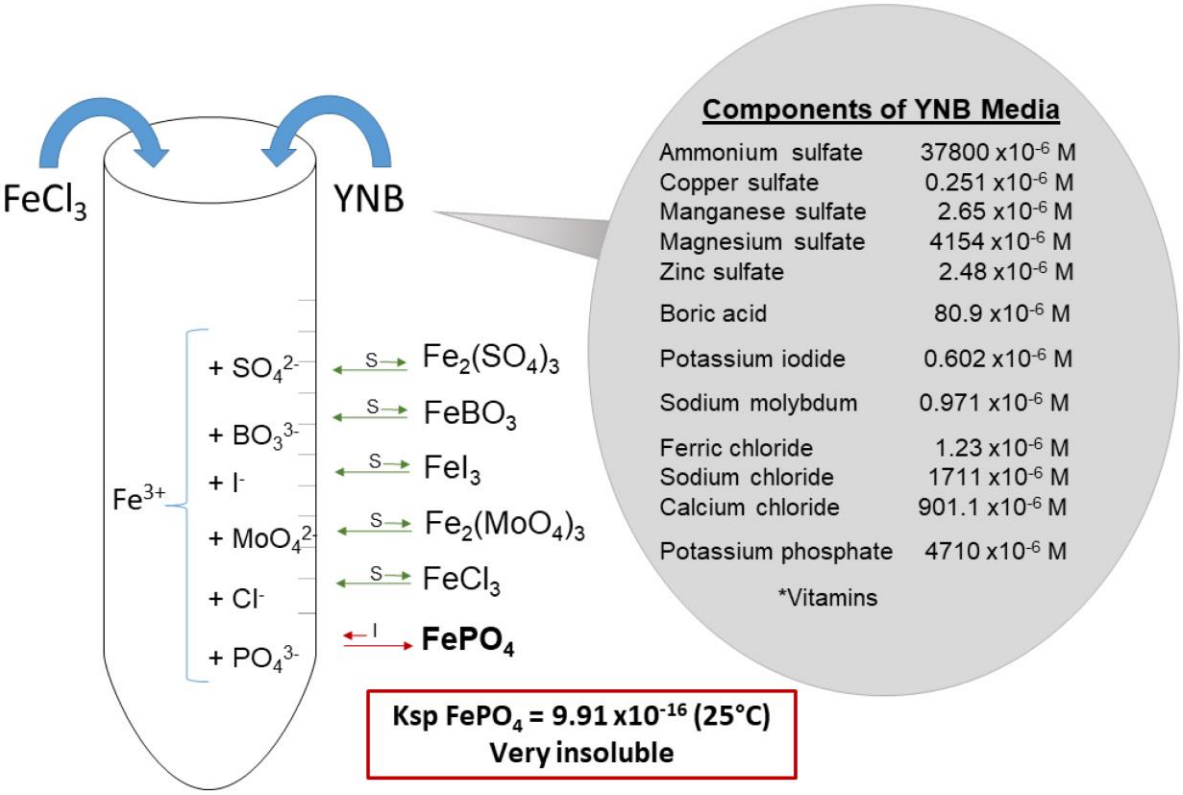

Figure 3.5. The ionic components of yeast growth media (YNB) and the effect of added iron.

Schematic representation of the chemical composition of the salts presents in yeast YNB media. The solubility of ferric (Fe^{3+}) with the different salts are shown as equation and the equilibrium constant for the solubility of $FePO_4$ is also shown.

Given that iron also commonly exists in two oxidation states (Fe^{2+} and Fe^{3+}), we also examined the effects of added ferrous iron. Although yeast is more resistant to ferrous iron, we can still see that 50mM iron leads to noticeable growth inhibition (Figure 3.2A). In addition, a noticeable precipitate is obvious when ferrous iron is added to YNB at 10mM, a concentration that does not lead to the inhibition of cell growth (Figure 3.2B). Thus, iron precipitation always precedes iron toxicity.

As a further control, we also examined the solubility of copper as well as it effects on yeast growth. Copper is like iron in that it is an essential micronutrient, it exists in different oxidation states (usually +1 and +2) as well as being a metal cation that is reported to be toxic and as well as apoptosis inducing [79], [114]. These results indicate that copper at 2mM leads to growth inhibition (Figure 3.3A) [3]. In contrast copper does not form any noticeable precipitation when added to YNB even at

concentrations as high as 10mM (Figure 3.3B). Thus copper toxicity, unlike iron toxicity, is not linked to limited solubility. It is noteworthy that 1.5 - 2mM is the range of copper sulphate that is reported to kill yeast [116]. The copper (as copper sulphate) content of YNB is 4×10^{-4} mM [103]. Thus, 20,000 fold excess of copper is required to observe negative effects of copper [112]. This is not surprising since intracellular content of copper like all essential nutrients are highly regulated by many different mechanisms [117], [118].

The limited solubility of iron is a commonly encountered problem especially when using complex solutions such as growth media [91], [95], [115]. Here we show that iron added (as ferric chloride) to YNB results in the dose dependent accumulation of a precipitate (Figure 3.1). The precipitate is detectable by weight with as little as 0.1mM added iron and appears to reach a plateau with 20mM added iron. The precipitate is also easily noticeable as the YNB media becomes visibly white and cloudy with 2mM added iron (Figure 3.1B).

A maximum of 4.5 mg (per ml of YNB) of precipitate accumulates after the addition of 20 mM ferric chloride. We examined the chemical composition of defined YNB media to determine possible ions that could serve to precipitate with added iron (Figure 3.5) [103]. Based on solubility, it is clear that phosphate is the most likely candidate [10]. Phosphate is insoluble with both ferric and ferrous iron and it is present in sufficient quantity (8mM) to account for the amount of precipitate formed with the added iron. In fact, the insolubility of iron-phosphate is so strong that it is exploited for a number of purposes. For example, phosphate can be used for iron removal in mining while iron can be used for phosphate removal in water purification systems [92], [119]. Iron is also used therapeutically in the treatment of hyperphosphatemia in patients with chronic kidney disease [120]. For plants, it is well known that excess phosphate in the soil will lead to iron deprivation (chlorosis) [113], [121]. The problem of phosphate-iron precipitation is commonly known for all cell culture media [91], [95], [115]. This has been clearly addressed in plant culture media but has not been so clearly addressed for yeast or mammalian cell culture systems. This is reflected in the literature where the problem of iron-phosphate precipitation has not been addressed in most if not all studies of iron toxicity using added excess iron [9]. In normal, cell culture media, iron

binding proteins like transferrin or chelators like EDTA or citrate are commonly used to prevent precipitation [91], [95], [115]. Although chelated iron can sustain cell growth, the chelated iron is not toxic [97]. It is known that iron and/or phosphate deprivation leads to growth inhibition and eventually to cell death [89], [122], [123]. Thus by simple deductive reasoning, it seems likely that the effects of excess added iron, is simply due to nutritional deprivation [124]. It should be kept in mind that iron mediated precipitation of phosphate may also lead to the co-precipitation of other ions such as copper and zinc [94]. Thus, it cannot be ruled out here that loss of these essential nutrients may contribute to the effect of excess iron on yeast cell growth.

Elevated levels of iron in plasma (iron overload) can occur for a variety of reasons including some conditions with uncontrolled dietary iron absorption (haemochromatosis) and others like thalessemia patients receiving frequent blood transfusions [9], [125]. This leads to the accumulation of free unchelated iron and which gives rise to pathological/toxicity effects. Chronic exposure to iron overload (years) leads to the formation of cellular vesicles containing high levels of iron and eventually cell death [9]. Cellular models have been developed in order to understand the cell's responses to high levels of extracellular iron. Older models of cultured mammalian cells fed high iron levels reflecting pathophysiological conditions for months on end mimic disease states and do accumulate large intracellular iron containing vesicles [9], [99]. These models are rarely used today. Instead, cultured cells including yeast and mammalian cells that expose cells to higher iron levels for shorter periods of time are more commonly used [9]. Iron mediated growth inhibition, cell death as well as the induction of regulated cell death processes like apoptosis and ferroptosis have been commonly reported for iron in all cells examined including yeast and cultured mammalian cells [9]. Although concentrations of iron in the mM range are commonly used to induce cell death, the accumulation of a precipitate has rarely been discussed. On the other hand, others have reported the use of iron in the μM range, concentrations that are normally non-lethal. Because of these limitations, it is not surprising that most (if not all) studies reporting on iron toxicity have combined iron with another sub-lethal stress to generate "iron toxicity" effects [100]. Serum starvation or deprivation, a known apoptosis inducing stress, is most commonly combined with sub-lethal iron in mammalian cultured cells [86], [102], [126].

Although the combined stresses are both required to induce a cell death it is more like synthetic lethality than iron mediated cell death [105]. In yeast, iron is more often studied in mutant cells that are characterized as iron supersensitive [100]. In our hands, the iron supersensitivity of some of these mutants is not restricted to iron but can also observed with other stresses like copper [3]. For example yeast mutants lacking vacuolar proton pump encoding VMA3Δ show pleiotropic phenotypes including a 10-fold increased sensitivity to both iron and copper compared to wild type cells [3], [127]. In addition, iron mediated cell death in VMA3Δ defective cells differs from the death that is seen in wild type cells since it can be prevented by anti-apoptotic genes. Taken together, it would appear that these studies are not examining iron mediated cell death but instead the cell deaths are more akin to synthetic lethality (lethality induced by two sub-lethal stresses) [105].

In conclusion, excess extracellular iron is not a direct chemical stress on a cell, its effect are instead mediated by the precipitation of iron with phosphate and the concomitant depletion of iron and phosphate as nutrients required to maintain growth and viability.

3.6 Acknowledgements

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3.7 Author Contributions

Data curation: Katie A. Miller, David R. Zhou, Michael T. Greenwood
Methodology: Katie A. Miller, David R. Zhou, Rawan Eid, Michael T. Greenwood
Supervision: Craig A. Mandato, Michael T. Greenwood
Writing – original draft: Katie A. Miller, David R. Zhou, Michael T. Greenwood
Writing – review & editing: Katie A. Miller, David R. Zhou, Michael T. Greenwood

Chapter 4 – Conclusions and Future work

Iron is both an essential micronutrient as well as a cyto-toxicant. Thus, iron is associated with two forms of human pathophysiologies, namely anemia because of inadequate iron and toxicity because of excess iron. Its ability to gain and lose electrons as it interconverts between ferrous (Fe^{2+}) and ferric (Fe^{3+}) forms is what makes it indispensable as an electron carrier in cellular processes such as respiration [9]. This same property makes iron toxic to the cell since free electrons can react with cellular constituents like hydrogen peroxide to produce highly toxic ROS [1], [9]. The Greenwood lab uses the yeast *S. cerevisiae* as a simplified model to study cellular responses to excess iron.

This research focused on the hypothesis that exogenous iron causes cell death by precipitating with an essential component of yeast media, leading to nutrient depletion [128]. Using spot growth assays, it was shown that iron is a dose-dependent inhibitor of yeast growth [128]. Correspondingly, the iron mediated accumulation of precipitation in yeast growth media was also found to increase in a dose dependent manner [128]. This was observed for both forms of iron, ferrous and ferric. Conversely, copper showed that it is a dose-dependent inhibitor of yeast growth but there was no formation of a precipitate, thus showing that iron is unique in its effects [128]. These observations demonstrate that iron mediated accumulation of a precipitate in YNB yeast growth media precedes the negative effects of iron on yeast cell growth.

Analysis of the chemical composition of yeast growth media suggests that the likely candidate responsible for the formation of the precipitate when iron is added is phosphate [10]. This is based on the fact that phosphate is present at sufficiently high concentrations to account for the formation of precipitate, and that it is highly insoluble with ferric (Fe^{3+}) iron, with a Ksp value for iron phosphate ($FePO_4$) of $9.91x10^{-16}$ (25ºC) (Figure 3.5). Additionally, the precipitation of $FePO_4$ has been extensively studied and used in many other industries, such as environmental wastewater purification systems or plant cell culture media [91], [95], [115]. The limitations of iron's solubility have been used to these industries advantage, but presently, not so clearly addressed in yeast or mammalian cell culture systems.

The use of iron and the possibility of precipitation in other cell culture systems, such as mammalian cells, is significantly under-reported. The observations of adding increasing concentrations of iron containing DMEM media exhibited that precipitation also occurs in mammalian cell culture media [128].

Ultimately, these results showing that exogenous iron caused precipitation, which preceded iron mediated growth inhibition, supports the hypothesis that iron toxicity is due to the depletion of phosphate, an essential micronutrient [128].

4.1 Future Direction

Possible studies could include measuring for phosphate in the supernatant using photometric phosphate detection approach, examining the specific elemental components of the precipitate using X-ray photoelectric spectroscopy (XPS), and looking at the viability of growing yeast cells re-suspended in different types of media, which lack essential nutrients, to investigate if nutrient depletion can induce PCD.

4.1.1 Phosphate determination

This could be accomplished by recreating the precipitation assay as described above in Chapter 3. The supernatant would be collected and the measurement of phosphate can be determined through colorimetric determination. Samples of the supernatant from different concentrations of added iron will be diluted and a known reagent for determining phosphate can be added, such as a Vanadate-molybdate solution [129], [130]. Spectroscopy can be used to measure the absorbance and the concentration of phosphate present can be determined through comparison of a standard curve [129], [130].

The principle of this determination is that in dilute phosphate solutions, ammonium molybdate reacts in an aid medium to form a heteropoly acid, molybdophosphoric acid [129], [130]. In the presence of vanadium the vanadomolybdophosphoric acid, yellow colour is formed [129], [130]. The intensity of the colour is proportional to the concentration of phosphate present in the solution. A standard curve, of a known phosphate solution, following Beer's law can be used to determine the unknown absorbance's of

each concentration of samples through comparison. It is hypothesized that the concentration of phosphate present in the supernatant will decrease as the concentration of iron increases. Thus, showing that iron precipitates with phosphate and it is being depleted from the yeast growth media.

4.1.2 Precipitate composition analysis

Another study could be to determine the exact elemental composition of the precipitate using electron spectroscopy for chemical analysis, XPS. This method is used to determine the elemental composition of a sample, as well as the chemical or electronic state of the elements. This can be achieved by recreating the precipitation assay as described above in Chapter 3 and collecting the solid precipitate. The prepared precipitate sample will be irradiated by x-ray beams, which will interact with the inner electron shell of the atoms present [131]. A photoelectron is released and the analyzer measures the kinetic energy of the photoelectron, which is the binding energy of the electron (shown as peaks) [131]. With the knowledge of binding energy, the element can be identified [131]. Limitations to this method include contamination, which can mask the surface structure of the elements [131]. In addition, it is unable to detect hydrogen.

4.1.3 Viability of yeast cells using media lacking essential nutrients

It is well known that the growth and maintenance of cells require specific nutrients. In this research, defined growth media, with all the nutrient requirements to grow and maintain yeast cells, was used [128]. Without all the required nutrients, cells will not be able to grow or replicate. Therefore, it would be of interest to investigate the response of yeast cells added to media lacking essential nutrients. The purpose of this would be to investigate the viability of cells suspended in media lacking a required essential nutrient. This would support the findings of this research which state that the formation of a precipitate, comprised of an essential nutrient from the growth media, facilitates cell death before the negative effects of iron has on the cell.

This can be achieved through a viability study using yeast cells and different types of modified yeast growth media, such as the commercial available yeast YNB media lacking single nutrients [132]. First, we would grow the yeast cells for 4 hours, centrifuge them, wash with water, and then re-suspend them in modified growth media. The type of media used could include normal YNB media (as a control), media without phosphate, media without iron, and media without sulphate. The Viability can be assessed by microscopic examination of the yeast cells, which are stained with a vital dye, Trypan blue [3]. The cells can be observed after different time increments, such as 30 min, 1 hour, 2 hours, 4 hours and 16 hours, to determine if PCD is induced. We expect growth to stop and the cells to remain quiescent and viable for prolonged periods upon the removal of most nutrients [78]. In contrast, we expect that phosphate removal will quickly cause cell death as seen in other cells [122]. Phosphate may be more crucial than most micronutrients since it is required in relatively large amounts, but also because it is required for many important biological systems, such as the production of ATP, the crucial energy molecule [89]. In addition, it is also an integral part of DNA molecules that are vital for maintaining and propagating cells [89].

This experiment would be interesting to determine if the absence of phosphate causes PCD. In the experiments described in this thesis are not sufficient to show the effects of phosphate depletion. This is because the formation of iron-phosphate may also cause the co-precipitation of other essential elements such as zinc and copper [94].

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