AN *EX VIVO* ERYTHROCYTE STUDY: THE PROTECTIVE EFFECTS OF ANTIOXIDANTS AGAINST THE TOXICITY OF HEAVY METALS IN CIGARETTE SMOKE

By

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Summary

Besides environmental exposure to metals, cigarette smoke either primary or secondary also contributes to metal exposure. This exposure in South Africa as well as in the rest of the world is increasing. Exposure to heavy metals such as lead (Pb), cadmium (Cd), chromium (Cr) and aluminium (Al) found in cigarette smoke can cause heavy metal toxicity, which includes the inhibition of antioxidant pathways via the depletion of antioxidant elements such as glutathione (GSH) and inhibition of antioxidant enzymes resulting in improper eradication of reactive oxygen species (ROS) which leads to cellular damage. A diet which is low in endogenous antioxidants such as polyphenols exacerbates this toxic effect. Supplementation with dietary antioxidants, if positive would be a cost effective, relatively simple method in helping to decrease the toxic effects of heavy metal exposure. Therefore the aim of this study was twofold, namely to determine the toxicity of metals commonly found in cigarette smoke using an erythrocyte *ex vivo* model and then to determine if antioxidants that are bioavailable can reduce toxicity. The methods used by this study are haemolysis assay, Fenton reaction, dichlorodihydrofluorescein diacetate (DCFH-DA) assay, Trolox equivalent antioxidant capacity (TEAC) assay, total flavonoid content (TFC) assay, Glutathione (GSH) assay and Scanning Electron Microscopy (SEM).

Pb was found to be the most toxic metal, causing 50% haemolysis (H50%) at a concentration of 16.00mM and toxicity was associated with echinocyte, type III formation. Pb was a poor catalyst of the Fenton reaction, but exposure of erythrocytes to Pb caused increased ROS formation. Pb did not bind GSH, however in erythrocytes it caused an increase in GSH levels. This implies that the oxidative effect of Pb, is not as a catalyst of the Fenton reaction or due to GSH binding. The observed effects may be due to the ability of Pb to inhibit antioxidant enzyme activity, resulting in an increase in GSH levels and subsequent accumulation of ROS. Catechin, gallic acid and 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) effectively reduced the oxidative effects of Pb. Catechin and gallic acid did not alter Pb induced increase in erythrocyte GSH levels. Catechin, gallic acid and Trolox bound GSH and reduced the amount of free GSH.

Cd induced H50% at a concentration of 33.83mM and the formation of spherocytes. The latter was similar to the effect observed with the oxidant AAPH. Cd catalysed the Fenton reaction and binds GSH, however in erythrocytes Cd did not cause an increase in ROS or alter GSH

levels. This implies that in the erythrocyte, Cd induced ROS formation, directly targets the cell membrane causing changes to membrane fluidity and morphology. Antioxidants did cause some inhibition of haemolysis and antioxidants quercetin and ascorbic acid inhibited the Fenton reaction and bound Cd. This metal antioxidant interaction caused a loss in the antioxidant activity of ascorbic acid but enhanced the activity of caffeic acid. Trolox reduced Cd – GSH binding.

At a concentration of 47.83mM Cr caused H50% and induced echinocyte type III formation. Cr catalysed the Fenton reaction and bound GSH in a manner similar to Cd. Likewise Cr did not cause an increase in ROS formation but did cause changes in GSH levels, similar to those seen with Pb. The higher Cr concentrations required for haemolysis and echinocyte type III formation implies that Cr may be slightly less toxic than Cd and Pb. Catechin and gallic acid reduced Cr induced haemolysis. In the Fenton reaction, quercetin and ascorbic acid scavenged hydroxyl radicals and this caused a loss in the antioxidant activity of ascorbic acid while enhancing the antioxidant activity of quercetin. Catechin and gallic acid reduced the ability of Cr to bind GSH.

Of all the metals investigated Al, was the least toxic, with H50% occurring at 81.26mM and was associated with echinocyte type I formation. Al was a poor catalyst of the Fenton reaction but did bind GSH. In *ex vivo* erythrocytes, Al did not induce ROS formation or changes in GSH levels. Catechin, gallic acid and Trolox reduced haemolysis. Trolox inhibited Al catalysis of the Fenton reaction. Al had no effect on the antioxidant activity of catechin, gallic acid and Trolox although metal antioxidant interactions enhanced the activity of quercetin and caused a loss in the antioxidant activity of ascorbic acid. Catechin and gallic acid caused an increase in erythrocyte GSH levels which was unaltered when erythrocytes were exposed to only Al.

In summary, some antioxidants, especially catechin, gallic acid, Trolox and ascorbic acid prevented metal induced cellular damage. The observed effects may be related to direct radical scavenging, GSH protection against metal binding or may be due to unknown membrane effects especially considering the effect of Trolox. Of concern is the adverse effect of these metals on the bioactivity of ascorbic acid.

Declaration

I, Akaashni Nareschandra Bhika hereby declare that this research dissertation is my own work and has not been presented for any degree of another University;

Signed: ……

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I dedicate this thesis to my parents. I would not be where I am today without your love, your belief in me and your endless support. Thank you for all that you do and for never giving up on me.

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List of Abbreviations, Symbols and Chemical Formulae

%	Percentage
^0C	Degrees centigrade
µg	Micrograms
µl	Microlitres
%H	Percentage Haemolysis
µg/µl	Microgram per microliter
µg/dL	Microgram per decilitre
µg/g	Microgram per gram
AAPH	2,2'-azobis(2-amidinopropane) dihydrochloride
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt
$ABTS^{\cdot+}$	ABTS radical
Al	Aluminium
AgNPs	Silver nanoparticles
ALA	Alpha-Linolenic acid
ALAD	δ-Aminolevulinate dehydratase
$AlCl_3$	Aluminium chloride
Al_2O_3	Aluminium oxide
ANOVA	Analysis of Variance
CAA	Cellular antioxidant activity
Ca^{2+}	Calcium ion
Cd	Cadmium
Co	Cobalt
CO	Carbon monoxide
COPD	Chronic obstructive pulmonary disease

Cr	Chromium
Cu	Copper
DCFH-DA	Dichlorodihydrofluorescein diacetate
DNA	Deoxyribonucleic acid
DTNB	5,5'-Dithiobis–(2-nitrobenzoic acid)
EDTA	Ethylenediaminetetraacetic acid
FDA	Fluorescein diacetate
Fe	Iron
GIT	Gastrointestinal tract
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSSG	Glutathione oxidized
GSH	Glutathione reduced
H_2O_2	Hydrogen peroxide
Hb	Haemoglobin
HCN	Hydrogen cyanide
Hg	Mercury
HMDS	Hexamethyldisilazane
$HO_2^{\bullet}$	Perhydroxyl radical
HOCl	Hypochlorous acid
HORAC	Hydroxyl radical averting capacity
Hrs	Hours
HT	Hydroxytyrosol
IARC	International Agency for Research on Cancer
IHD	Ischaemic heart disease
Iso PBS	Isotonic phosphate buffered saline
KCl	Potassium chloride
K_2CrO_4	Potassium chromate

Kg	Kilogram
KRA	Kirsten rat sarcomas
L	Litres
MDA	Malondialdehyde
mM	Millimolar
M	Molar
Mg	Milligram
MPO	Neutrophil myeloperoxidase
NAC	N-acetlycysteine
Ni	Nickel
Nm	Nanometre
NO	Nitric oxide
$^1O_2$	Singlet oxygen
O_2^-	Superoxide anion
$OH^\bullet$	Hydroxyl radical
OH^-	Hydroxyl anion
$ONOO^-$	Peroxynitrite
P-value (p)	Probability value
PAH	Polycyclic aromatic hydrocarbons
Pb	Lead
PBS	Phosphate buffered saline
pH	Logarithmic scale for measurement of the acidity or alkalinity of an aqueous solution
ROS	Reactive oxygen species
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
RT-PCR	Reverse transcription polymerase chain reaction
SCAL1	Smoke and Cancer Associated LncRNA 1

SDS	Sodium dodecyl sulphate
SEM	Scanning electron microscopy
SH	Sulfhydryl
SOD	Superoxide dismutase
TB	Tuberculosis
TOC	Tocopherols and tocotrienols
TP53	Tumour protein 53
Trolox	6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
Zn	Zinc

CHAPTER 1: INTRODUCTION

'If smokers had the same death rate as non-smokers, 58% of lung cancer deaths, 37% of COPD deaths, 20% of tuberculosis deaths and 23% of vascular deaths would have been avoided in South Africa. Approximately 8% of all deaths in South Africa (more than 20 000 deaths per year) were caused by smoking.'[1]

These statistics were reported in a study conducted by Sitas[1] *et al*. (2004) which identified that smoking annually contributes significantly to deaths in South Africa.[1] In this population smoking is directly linked to several diseases including chronic obstructive pulmonary disease (COPD), lung cancer and ischaemic heart disease (IHD).[2] In addition cigarette smoke worsens conditions such as asthma and tuberculosis (TB). The main components of cigarette smoke that contribute to the above-mentioned diseases are gasses such as carbon monoxide (CO), hydrogen cyanide (HCN), nitrogen oxides, hydrocarbons such as benz(a)pyrene as well as toxic heavy metals, aluminium (Al), cadmium (Cd),chromium (Cr), copper (Cu), lead (Pb), mercury (Hg), nickel (Ni) and zinc (Zn).[3] Cigarette smoke interferes with the metal homeostasis of the body which results in the development of several diseases.[4] The metals found in cigarette smoke are also catalysts of the Fenton reaction. They inhibit antioxidant components and/or enzymes which alter normal physiological processes resulting in oxidative stress which leads to inflammation and damage to cells, tissue and organ systems. This leads to cancers, cardiovascular and degenerative disease as well as accelerated aging.[4]

Animals and humans have many defence mechanisms which limit the levels of reactive oxygen species (ROS) and the levels of subsequent damage. These defence systems include endogenous antioxidant molecules and enzymes and exogenous dietary antioxidants. To counteract the effects of heavy metal toxicity due to cigarette smoking and/ or other sources such as environmental exposure, exogenous dietary antioxidants are believed to play an important role in counteracting the oxidative effects of heavy metals. This could be by inhibiting the Fenton reaction, by directly binding the metal or by preventing the depletion of glutathione (GSH).

The aim of this exploratory study was to determine the oxidative toxicity of cigarette associated metals, Pb, Cd, Cr and Al in an *ex vivo* erythrocyte model. In addition the ability of common, bioavailable antioxidants to reduce metal induced oxidative toxicity was also determined.[5]

CHAPTER 2: LITERATURE REVIEW

Tobacco represents the single most preventable cause of disease and death in the world today.[6] In a study conducted by Reddy[7] *et al*. (1996) it was predicted that within a few decades of the study being completed that tobacco use and cigarette smoking would become one of the most significant causes of premature death in South Africa. In this publication, it was emphasised that there was an urgent need to develop multilevel interventions that target tobacco use and the smoking of cigarettes.[7] The prevalence of smoking in South Africa in the year 2004 was similar to the rates found amongst other international based population studies conducted within a similar time frame. This study found that approximately 54.8% of males and 15.8% of females in South Africa are smokers.[1] Walbeek[8] *et al*. (2002) reported that between 1993 and 2000 there was a narrowing of the 'gender prevalence gap' between males and females. This narrowing gap was seen not just in South Africa but was consistent with international studies, indicating that worldwide more females now smoke.[8] In a South African based study, Sitas[1] *et al*. (2004) reported that smoking cigarettes significantly increases the risk for diseases such as TB, lung cancer, upper aerodigestive cancer and IHD.

2.1 TOBACCO AND ASSOCIATED HEAVY METALS

Tobacco plants *(Nicotianan tabacum)* are grown in almost every country in the world and approximately 80% of the world consumption of tobacco is through cigarette smoking.[9] Plants are exposed to different types of xenobiotics which is either deliberate in cases of pesticides and fertilizers or accidental in cases of polluted soil, water and air.[10] Tobacco can easily accumulate a number of heavy metals in the stalk and the leaves.[9,11] Concentrations of heavy metals are higher in the older leaves compared to the younger leaves and stalks. The phosphate fertilizers used in the cultivation of tobacco generally also contains high concentrations of heavy metals.[11] When the harvested tobacco is processed into cigarettes these toxic elements pass from the tobacco to the cigarette smoke and the environment as secondary smoke. Cigarette filters retain only a small portion of the elements. The environmental pollution due to secondary smoke does not consist solely of the smoke exhaled by the smoker, but also the side stream of smoke that is released by the burning cigarette. This side stream smoke is inhaled by non-smokers and usually contains high concentrations of many of the toxic substances including heavy metals.[9] The four most common types of heavy metals found in cigarette smoke are Pb, Cd, Cr and Al and these metals were used in this study and the known effects thereof were discussed in greater detail.

2.2 REACTIVE OXYGEN SPECIES AND OXIDATIVE STRESS

Free radicals can be defined as molecules or molecular fragments that contain one or more unpaired electrons in their outer orbital. This unpaired electron usually makes these molecules highly reactive and they can either donate or accept an electron and consequently, these molecules are less reactive.

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are products of normal cellular metabolism and play a dual role as both harmful and beneficial species. The beneficial effect of ROS and RNS occurs at low concentrations and plays an important role in cellular functioning and preventing infection by activation of the innate immune system. However excess ROS formed by pro-oxidative enzymes, lipid peroxidation, inflammation, smoking, air pollution and other harmful stressors[12] has an adverse effect on cellular functioning. Oxidative stress occurs when the critical balance between ROS and antioxidants is disrupted, which then leads to a number of diseases.[13] This takes place in biological systems when there is an over production of ROS/RNS and/or a decrease or deficiency of enzymatic and non-enzymatic antioxidants.

ROS includes free radicals like the superoxide anion radical (O_2^-), perhydroxyl radical ($HO_2^\bullet$), hydroxyl radical ($OH^\bullet$), nitric oxide (NO) and other species such as hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), singlet oxygen ($^1O_2$) and peroxynitrite ($ONOO^-$).[14] Radicals that are derived from oxygen are the most important physiologically relevant radicals. Molecular oxygen has two unpaired electrons in its outer orbital making it extremely unstable. Most oxygen that is taken up by human cells is reduced to water by means of mitochondrial cytochrome oxidase. The intermediate step in the reduction of oxygen produces O_2^-, H_2O_2 and $OH^\bullet$.[15]

The O_2^- is formed either through metabolic processes of oxygen activation or through physical irradiation. The superoxide anion is seen as the “primary” ROS, and can interact with other molecules to produce “secondary” ROS. This can occur either directly or by enzyme or metal catalysed reactions.[16] $OH^\bullet$ is extremely reactive, and is formed via the metal catalysed Fenton reaction. In the Fenton reaction, ferrous iron (Fe) (II) is oxidised by H_2O_2 to ferric Fe (III), $OH^\bullet$ and a hydroxyl anion (OH^-) is then formed as shown in the reaction below.[17]

$$\mathbf{Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^\bullet + OH^-}$$

Numerous other metals such as Cu, Cr and cobalt (Co) can also act as catalysts as shown in the reaction below, resulting in the formation of hydroxyl radicals.

Metal (oxidised) + H_2O_2 → Metal (reduced) + $OH^{\bullet}$ + OH^-

2.3 HEAVY METALS IN CIGARETTE SMOKE AND OXIDATIVE EFFECTS

Humans are continuously being exposed to heavy metals such as Pb, Cd, Cr and Al. Sources of exposure include contaminated food, water, soil and air.[18] The consequences of exposure are structural damage to proteins, membrane lipids and nucleic acids that result in altered cellular structure and function which can then lead to diseases such as COPD, cardiovascular disease and lung cancer.[19,20] A common mechanism that is involved in the development of these diseases is the induction of oxidative damage. Toxic heavy metals increase the production of highly reactive ROS and can also inactivate antioxidant enzymes and elements such as GSH, and decrease the availability and accessibility of antioxidants in the body to neutralise ROS production. This in turn results in oxidative damage and disease.[21] Several *in vitro* studies have been conducted and show that alveolar leukocytes and macrophages from smokers spontaneously release elevated amounts of oxidants such as $O_2^{\bullet}$ and H_2O_2 when compared to those from non-smokers.[22-25]

2.3.1 TOXICITY OF LEAD

Pb is without a doubt one of the oldest occupational and environmental toxins around, and evidence of Pb toxicity can be dated back to Roman times where people suffered from *Colica Pictomum,* a deadly disease that causes severe colic, paralysis and other dysfunctions of the central nervous system. This disease was later identified as chronic Pb disease, which was associated with ingesting Pb-laced wines. The custom of treating and sweetening sour wines with Pb-containing flavourings is traced back to the Romans.[26,27]

In the last few decades the detection and prevention of Pb toxicity has been a priority in international public health and several measures have been taken to decrease the exposure of Pb. Some of these measures include: decreasing the amount of Pb in paint, removing Pb from petrol thus producing unleaded petrol and banning of Pb solders in food cans. These measures have reduced Pb exposure. However Pb toxicity is still a major problem throughout the world affecting both children and adults.[28] Even though the incidence of severe Pb exposure has decreased in many countries, occupational Pb exposure is still common. Workers are exposed to Pb in several industries including the assembly of motor vehicles, battery manufacturing and recovery, Pb mining and smelting, production of Pb alloys and in the production of glass, paint, plastics and ceramics.

The concern regarding Pb is that recent research has shown that it has adverse health effects at much lower concentrations than previously accepted.[28] In the 1960's a blood Pb level of 60µg/dL was considered safe.[28] With research and new findings, the acceptable blood Pb level was reduced in 1985 to 25µg/dL, and was reduced further in 1991 to 10µg/dL. In spite of this, indications are that even lower levels can have toxic consequences.[28]

Pb is a persistent environmental toxin that has a negative impact on many systems in the body and these include the haematological, neurological, gastrointestinal, reproductive, circulatory, immunological and urinary systems which when affected can lead to associated pathologies.[21,29] Low Pb levels have also been shown to cause cognitive dysfunction and neurobehavioral disorders.[21] The International Agency for Research on Cancer (IARC) has classified Pb as a human carcinogen based on adequate data from animal studies and is classified as a weak human carcinogen that most likely contributes to lung cancer, stomach cancer and gliomas.[30]

Pb can be absorbed though ingestion, inhalation and small amounts can be absorbed through the skin. Up to 50% of inhaled inorganic Pb can be absorbed through the lungs. Adults absorb between 10-15% of Pb found in food through the gastrointestinal tract (GIT), while children can absorb up to 50% of Pb through the GIT. Pb found in blood binds to erythrocytes and elimination of blood bound Pb is slow and via the urine. Pb also accumulates in the skeleton. The half-life of Pb in the blood is approximately one month and approximately 20-30 years in the skeleton.[30] Inorganic Pb does not cross the blood brain barrier in adults, however in children the blood brain barrier is not fully developed and consequently Pb can cross into the brain which can lead to brain damage and other neurological pathologies.[30]

Smoking is not the main source of Pb uptake by humans, however the involvement and contribution of smoking to the total Pb load in humans has become increasingly relevant over the years.[4] The average concentration of Pb in filter-tip cigarettes is approximately 2.4µg/g. Of this 2.4µg/g of Pb, roughly 6% passes into mainstream smoke which is inhaled by the smoker.[4,9] In a study conducted by Chiba and Masironi[9] (1992) it was reported that smokers and former smokers have higher blood Pb levels than non-smokers.[9] Passive smoking plays a significant role in Pb exposure to children. In a study done by Andren[31] *et al*. (1988) blood Pb levels in children with parents that smoke were much higher than in children of non-smokers.[31]

Increasing exposure to Pb is seen to cause an increase in both hypochromic and normochromic anaemia.[21,32] A main target of Pb toxicity is the haematological system and Pb can inhibit heme and haemoglobin (Hb) synthesis and adversely affects the morphology and survival of erythrocytes. Pb exposure can also cause Hb oxidation which can also lead to erythrocyte haemolysis. The mechanism behind this reaction is Pb-induced inhibition of δ-

aminolevulinate dehydratase (ALAD) activity. ALAD is the enzyme most sensitive to Pb toxicity and inhibition of this enzyme leads to decreased heme production. With decreased heme production, elevated levels of alpha-linolenic acid (ALA) are found in both circulating blood and excreted urine of Pb-exposed subjects.[21,32] There have been several studies conducted concerning accumulated ALA and ROS generation. These studies show that the accumulated ALA generates H_2O_2 and O_2^-. The excess ALA also reacts with oxyhaemoglobin which results in the formation of $OH^{\bullet}$.[21,32-34] As ALA becomes more oxidised it forms 4,5-dioxavalenic acid. This acid is a probable genotoxic compound and is a potential mechanism for the metal-dependent DNA carcinogenicity of Pb.

As erythrocytes have a high affinity for Pb, the erythrocyte model has been used to evaluate Pb toxicity. Following *ex vivo* exposure to Pb it was found that the erythrocytes become more vulnerable to osmotic and mechanically induced cellular damage.[18] Other studies have found a direct correlation between the toxic effects of Pb and Pb-induced oxidative damage in erythrocytes. [18] Yiin and Lin[35] (1995) reported that Pb causes lipid peroxidation in essential unsaturated fatty acids which is also a probable mechanism of the toxic effects of Pb, which causes damage to the polyunsaturated fatty acids found in cell membranes. Kasperczyk[36] *et al*. (2015) investigated the effect of occupational Pb exposure on lipid peroxidation, protein carbonylation and plasma viscosity. Malondialdehyde (MDA) and protein carbonyl levels were found to be significantly increased, whereas protein and protein sulfhydryl levels were significantly decreased. In this study, it was concluded that Pb exposure leads to oxidative stress that results in lipid and protein damage.[36] These studies identified that Pb caused membrane damage, altered membrane lipid composition and structure altering membrane integrity, permeability and function.[32,37]

Besides its effect on ALAD and the erythrocyte membrane, several studies have shown that heavy metals cause alterations in antioxidant enzyme activities. Pb is shown to both increase and suppress blood levels of superoxide dismutase (SOD), catalase, glutathione peroxidise (GPx) and glutathione reductase (GR).[21,38] Pb has several cellular targets and the effect on these targets either produces ROS or inhibits antioxidant systems which leads to an imbalance in the pro-oxidant/ antioxidant cellular equilibrium resulting in oxidative damage.[32] The consequence thereof in erythrocytes is direct enzyme inhibition, altered protein function as well as ROS induced changes in lipid and protein structures (Figure 2.1). The effects of Pb are not limited to the effects on erythrocytes as described in this section. Oxidative effects in other cell types can lead to altered deoxyribonucleic acid (DNA) structure and mutations leading to the development of cancer (Figure 2.1 and Section 2.4.2).

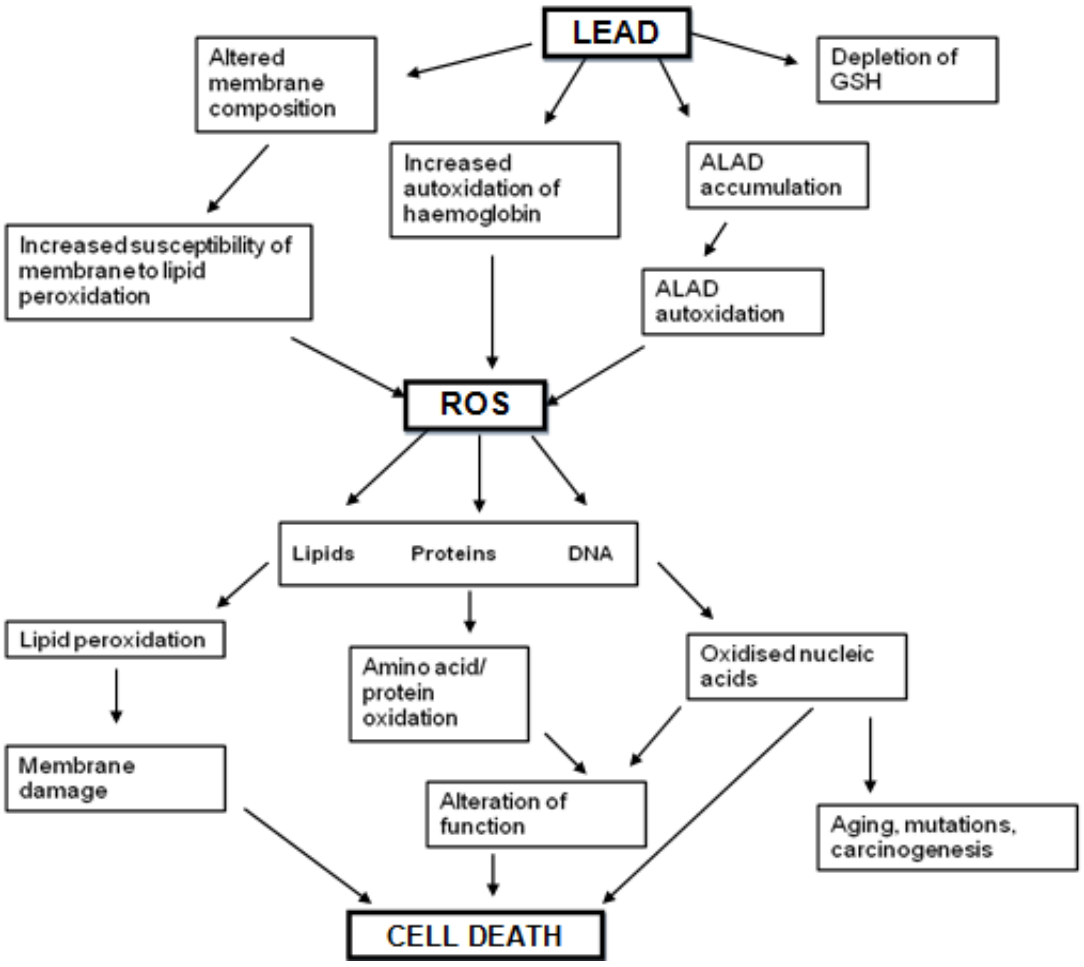


Figure 2.1: Flow diagram summarising the mechanisms of Pb mediated oxidative damage (Adapted from Gurer and Ercal[32], 2000).

2.3.2 TOXICITY OF CADMIUM

Cd has been recognised as an environmental hazard for many decades.[39] The risk of Cd exposure to the general public and to environmentally exposed populations was emphasized after the 1930's when a case of Cd poisoning in the Jinzu River in Japan was reported. Water was contaminated with very high levels of Cd and rice plantations were irrigated with this water. This lead to mass exposure to Cd which resulted in the development of Itai-Itai disease which is a combination of osteoporosis and osteomalacia, which results in weak deformed bones and extreme pain. This was exposure on a massive scale and consequently worldwide studies were undertaken to monitor Cd levels and to keep these levels to the minimum.[40] Even though this monitoring occurs, public exposure to Cd from various sources is still common.[40]

Like Pb, Cd is present in air, dust, soil and water in various amounts and each can act as a route of exposure in humans and animals. Even though Cd is a non-essential element it is still present in almost all food sources.[41] The concentration of Cd varies according to the food type

and the level of environmental contamination. Seafood such as molluscs, crustaceans and oysters have a high Cd content. Food from plant sources can also contain high Cd levels and Cd can bio-accumulate in animal sources such as meat and dairy products.[39] Cd can be absorbed in the body by ingestion (a few percentage units) or inhalation (10-50%). Once in the body, Cd is transported by the erythrocytes and proteins.[39,42] Cd is efficiently retained mainly in the kidney with a biological half-life of approximately 10-30 years. In the kidneys, Cd exposure has been seen to cause irreversible renal tubular and glomerular damage.[42]

Cd affects several systems in the body and is implicated in several clinical disorders such as renal dysfunction, bone disease and has been linked to the development of breast, prostate, colon, rectal, kidney and lung cancer. Cd has also been classified by IARC as a known human carcinogen.[43]

Cd is found naturally in ores, as well as PVC paints, in rechargeable Ni-Cd batteries, anti-corrosion agents, phosphate fertilizers, industrial emissions, contaminated food sources and cigarette smoke.[30,44,45] The main source of exposure for smokers is tobacco smoke while for non-smokers it is dietary.[30] In food sources, contaminated soil leads to an increased uptake of Cd by crops and vegetables and subsequent bio-accumulation in dairy and meat products.

Cd accumulates in tobacco plant leaves in unusually high concentrations.[9] This is because tobacco plants have an unusual ability to absorb and store Cd.[9] The concentration of Cd found in cigarettes ranges between 0.5 to 3.5µg/g, with an average of 1.7µg/g per cigarette.[9] The results of several studies show that blood Cd concentrations are elevated with increased smoking.[9] With smoking, Cd is converted to CdO which is then inhaled. Approximately 10% of the inhaled smoke deposits in the lungs and 20-50% of this is transferred to the circulation.[4] Cd exposure is known to cause harmful effects in both humans and animals. The mechanism of damage is not entirely known, however oxidative stress has been identified as a probable mechanism of action.

Cd can act as a catalyst of the Fenton reaction which converts H_2O_2 into free radicals[17] (see Fenton reaction in Section 2.2). These free radicals then cause oxidative stress. In addition Cd inhibits the antioxidant enzymes, SOD, GSH, and catalase.[43,46] Cd affects SOD by binding to the amino acid residue histidine 74 of the SOD enzyme which causes inactivation of the enzyme.[47] Cd binds the thiol group of GSH thereby altering its structure and function.[43] Loss of this cellular antioxidant molecule leads to increased accumulation of ROS such as $O_2^{\bullet-}$, H_2O_2 and $OH^{\bullet}$. This then leads to lipid peroxidation, damage to the DNA, oxidation of proteins and may eventually lead to cellular dysfunction and/or death. In erythrocytes, this destruction causes haemolysis.[51] In rats Cd exposure resulted in increased ROS formation and lipid peroxidation which in turn lead to haemolysis and anaemia.[51]

Furthermore, Cd displaces Fe and Cu in different cytoplasmic and membrane proteins, for example in ferritin and apoferritin. This causes an increase in the amount of unbound, free Fe and Cu ions which can then also catalyse the Fenton reaction with the formation of $OH^{\bullet}$ radicals.[17,48]

Besides the blood, exposure to Cd can also cause oxidative damage to a variety of tissues and organs such as the lungs, kidneys, bone, central nervous system, reproductive organs and heart.[17]

2.3.3 TOXICITY OF CHROMIUM

Cr has been used in numerous industries for over a century and its excessive exposure in the work place has shown that it is an acute irritant, a carcinogen and an allergen to humans and animals.[52] Cr is found in several oxidation states. Cr(VI) ion has been established as the main cause of chrome toxicity, and the Cr(III) ion has been established as more of an irritant than a carcinogen or allergen. Cr has a very high affinity for erythrocytes thus, once Cr(VI) crosses the erythrocyte membrane, it is reduced to Cr(III) which binds to the cellular components and in this form Cr accumulates in erythrocytes.[52,53] Cr(VI) compounds are more toxic as Cr(VI) can easily cross the cell membrane while Cr(III) cannot cross the cell membrane, although once across the cell membrane Cr(III) is the major toxicant.[53]

Cr like the other metals can be found in air, soil, dust and water and each source can act as a route of exposure in humans and animals.[52] Cr is used in several industries including chromate production, metal plating, manufacturing of alloys, metal welding and forming processes.[52] The general public is exposed to Cr through contaminated food and water sources, and cigarette smoke. Cr(III) is an essential element in the body and is needed in minute amounts for normal glucose metabolism and functioning.[54] The estimated safe and sufficient daily dietary intake for Cr is between 50 and 200µg.[55] Cr deficiency has been noted in a number of patients and results in impaired glucose tolerance, glycosuria and elevated insulin and glucagon levels, which mimic symptoms similar to those of non-insulin-dependent diabetes mellitus and associated cardiovascular disease.[54,55] In excessive amounts Cr is toxic to humans and animals. Cr (VI) was declared a human carcinogen in 1980 by the IARC.[56] Exposure is via ingestion, inhalation or dermal absorption. This metal is seen to accumulate in epidermal tissue such as hair and nails, bones, blood, liver, kidney, spleen, lungs, large intestine and muscles.[57] Several studies have been conducted on Cr toxicity and the results of these studies indicated that Cr causes pathological and anatomical changes in the lungs, kidneys and liver of humans and animals. Cr has been found to affect the respiratory system by causing perforation of the nasal septum, nasal bleeding, conjunctivitis, lung cancer, hyperaemia, erosion and inflammatory changes in the respiratory system in patients after

inhalation of Cr compounds. Acute Cr exposure also results in renal tubular necrosis and kidney damage.[57-59]

Beside food sources, in the general population cigarette smoke is a key source of Cr exposure. Based on several studies the Cr levels found in main stream cigarette smoke ranges between 0.0002-0.5µg per cigarette.[4] Cr accumulates in tissue, especially in the lung. The average concentration of Cr in lung tissue of smokers is 4.3µg/g compared to 1.3µg/g in non-smokers. This concentration increases with age and smoking time. The presence of Cr in cigarette smoke is also linked to the development of emphysema.[4,60,61]

With the reduction of Cr(VI) to Cr(III) several free radicals are generated and high levels of Cr-DNA-adducts are formed which results in mutations and DNA damage. Intermediate oxidative states of Cr are also suggested to cause genotoxicity, carcinogenicity and oxidative stress, either through direct mechanisms or through reactions like the Fenton reaction with the formation of ROS.[17,62,63] Cr(III) can be reduced to Cr(II) by the Fenton reaction, where H_2O_2 and free radicals are formed. Thus, to sum up oxidative stress when dealing with all the different oxidative states of Cr; Cr reduction leads to the production of numerous free radicals such as O_2^- and $OH^{\bullet}$.[15] These free radicals react with the cell membrane and DNA bases causing cellular damage.[17,64]

Although erythrocytes do not have DNA or organelles such as mitochondria, Cr also adversely affects erythrocytes leading to apoptosis-like cell death called eryptosis which is characterised by cell shrinkage and scrambling of the plasma membrane. Eryptosis can occur as a result of an increase in cytosolic Ca^{2+} activity, ATP depletion or ceramide formation. Exposure of erythrocytes to ≥ 10µM Cr(IV) for 48 hours (hrs) caused an increase in cytosolic Ca^{2+} levels associated with the depletion of cytosolic ATP, leading to eryptosis and scrambling of the cell membrane resulting in haemolysis.[65] Exposure of rats to Cr(IV) for one and two weeks caused increased erythrocyte haemolysis, lipid peroxidation, carbonyl formation as well as a decrease in GSH and ascorbic acid levels.[66]

2.3.4 TOXICITY OF ALUMINIUM

Over one hundred years ago Siem and Dollken conducted the first studies on the neurotoxicity of Al. Since then numerous studies have been conducted and Al has been established as a potent neurotoxin.[67] Al has a long history of being used for purification of water and in medications. The Romans used Al salts to purify water, and in the Middle Ages this metal was mixed with honey and used as a treatment for ulcers. This metal is still used today in water purification and Al salts are still used in medications such as antacids and aspirin.[68] Al is also

used in numerous products such as food preservatives, colouring agents, some antiperspirants and foil packaging. Other sources of exposure are industrial exposure to Al containing dust, and Al in cigarette smoke.[69]

Like most other metals Al is found in, air, dust, soil and water sources with the latter being the major cause of food contamination. Al can be absorbed into the body by inhalation, ingestion and dermal absorption.[68] Al chemistry is similar to the chemistry of Fe, thus Al is transported in the body by the Fe-binding protein transferrin. Al accumulation in the brain is linked to its neurotoxicity and a possible link has been reported between the Al accumulation in the brain and Alzheimer's disease, due to Al induced neurofibrillar degeneration. Elevated levels of Al are found in the degenerating neurons of patients with Alzheimer's disease.[70]

The Al content of cigarette smoke is relatively high. However, several studies have shown that blood, urine and plasma levels of Al are not elevated when comparing smokers to non-smokers.[4,60] Although elevated Al levels are found in the brain of Alzheimer's patients no direct link between smoking and Alzheimer's disease has been found.[4]

Oxidative events have often been linked with Alzheimer's disease and whether the presence of these oxidative events is the cause or the consequence of ROS formation during neurodegeneration is unknown. Al cannot form ROS directly but can potentiate the formation of these species by Fe and Cu. A hypothesis is that Al may bind to these metals and alter their ability to promote metal-based oxidative reactions. Consequently, these metal colloidals have a pro-oxidant effect by catalysing the Fenton reaction resulting in oxidative damage.

Intraperitoneal injection of Al gluconate administered over a period of three weeks increased both the rate of ROS formation and the levels of GSH in cortical brain tissue of rats.[71] Al has also been found to facilitate Fe-mediated oxidation in biological membranes. Al is also capable of causing damage to erythrocytes such as increased lipid peroxidation and inhibition of the activity of erythrocyte antioxidant enzymes, SOD, catalase and GPx. Al also causes morphological changes to the surface of the erythrocyte membrane.[72] The erythrocytes of rats that were exposed to 50mg/kg body weight of Al in drinking water were found to have increased MDA and H_2O_2 levels and decreased GSH levels. The activity of SOD, catalase and GPx were also decreased in these rats.[73]

2.4 DISEASES ASSOCIATED WITH SMOKING

There is overwhelming evidence from research done over the years that proves that smoking and several components found in cigarette smoke at least partially contributes to the

development of various life-threatening diseases such as emphysema, cardiovascular disease and cancer.[74] The role of cigarette smoke and oxidative damage in the development of COPD and cancer will be discussed in greater detail.

2.4.1 CHRONIC OBSTRUCTIVE PULMONARY DISEASE

COPD is a debilitating disease and although in most instances this disease is preventable and treatable, the incidence of COPD continues to rise due to the worldwide epidemic of smoking.[74] COPD is characterised by the development of a persistent obstruction or limitation of airflow which cannot be entirely reversed. The clinical syndrome of COPD includes different disease conditions such as chronic obstructive bronchitis which causes obstruction of the small airways, and emphysema which is characterised by enlargement of airspaces and destruction of lung parenchyma, decrease in lung activity and closing of the small airways.[75,76]

The most common onset and development of COPD occurs between the ages of 45 and 60 years in smokers and is the fourth most common cause of death in the United States.[77,78] Clinical features in patients with COPD are based on the occurrence of a productive cough, wheezing, and shortness of breath.

The main tissue associated features of this disease are hypertrophy and hyperplasia of the submucosal glands, varying amounts of inflammatory cell infiltration of the mucosa and smooth muscle hyperplasia as well as numerous inflammatory changes in the respiratory bronchioles. Upon physical examination of the patient, air flow obstruction may be noted.[77,78] Patients with COPD may also present with the following systemic manifestations and comorbidities: skeletal muscle wasting, loss of fat free muscle, lung cancer, pulmonary hypertension, ischaemic heart disease, congestive heart failure, osteoporosis, anaemia, diabetes, metabolic syndrome, obstructive sleep apnoea and depression.

Cigarette smoking is the most significant and well documented risk factor for COPD. Genetic $alpha_1$-antitrypsin deficiency is also associated with an increased susceptibility to COPD. Air pollution and chronic childhood infections are additional risk factors for COPD.[77,78] Other risks include airway hypersensitivity, occupational exposure to dusts and exposure to oxidant gases often found in low socioeconomic environments.[77]

Patients with COPD have a 3-4 times increased risk of developing lung cancer than smokers with normal lung function. Lung cancer is a common cause of death among COPD patients.[79] The sources of increased oxidative stress in COPD patients is due to the presence of oxidants in cigarettes or those produced following absorption of metals found in cigarette smoke. It is

also due to disease related increase in the number of inflammatory leukocytes and alveolar macrophages in both the circulation and alveolar spaces.[80-82] Neutrophil myeloperoxidase (MPO) levels are increased and this correlates with pulmonary dysfunction. The depletion of the body's antioxidant systems and/or a dietary deficiency of these antioxidants is also a contributing factor to oxidative stress. Carcinogenic hydrocarbons and oxidative damage due to the presence of heavy metals can act independently or synergistically in the development and progression of lung cancer. This indicates that oxidative stress mediated by increased levels of MPO in the neutrophils plays a role in inflammation of the lung.[80,81,83] Cigarette smoking also increases RNS formation which leads to the nitration and oxidation of plasma proteins. *In vitro* exposure of blood to cigarette smoke results in increased erythrocyte lipid peroxidation and protein carbonylation in the plasma.[84] The antioxidant defence systems of the cells are also altered with cigarette smoking. GSH is the predominant form of glutathione with the oxidized form, glutathione disulfide (GSSG) being a marker/indicator of a poor oxidative status. In the sputum of patients with COPD, GSH levels are reduced and GSSG levels are increased.[80] This indicates that oxidative stress plays a major role in the pathology of COPD's, and cigarette smoke is a contributing factor.

2.4.2 LUNG CANCER

Lung cancer is the second most common type of cancer found in both males and females in the United States and is the number one cause of cancer-related death.[85] Worldwide, lung cancer kills over one million people every year.[86] Extensive studies revealed that cigarette smoke is the leading cause of lung cancer.[87] As with other cancers, lung cancer is thought to arise after a progression of pathological changes or preneoplastic lesions in the bronchial epithelium.[88] Most types of lung cancers are associated with multiple gene alterations and preneoplastic bronchial lesions. The role of tobacco smoking in the development of lung cancer is recognized for squamous and small cell type lung carcinoma. Studies also show a greater risk of adenocarcinoma among smokers than non-smokers.[89] Patients suffering from COPD's also have an increased risk of lung cancer. This link is probably due to the elevated amounts of inflammation and oxidative stress in COPD. Pro-inflammatory cytokines that are present in COPD can also promote the formation of tumours which increases the rate of cell growth and metastases. Transcription and growth factors are also altered in patients with COPD and may contribute to their susceptibility to lung cancer.[79]

The mechanisms of carcinogenic metabolic activation and detoxification have been extensively researched[79] and include ROS induced lipid peroxidation, protein carbonylation and DNA nicking such as single-strand DNA breaks.[87,90]

Polycyclic aromatic hydrocarbons (PAH) which are also found in cigarette smoke are a large group of structurally related molecules formed during the incomplete combustion and heat associated breakdown of organic matter. Many PAHs are carcinogenic in humans and exposure to these PAHs includes air pollution, occupational exposure in Al production, roofing, paving with tar and cigarette smoke.[91]

The metabolic activation of absorbed PAH, leads to PAH diol epoxide metabolite formation which results in the formation of metabolic adducts that bind covalently to DNA.[91] If these adducts escape cellular repair mechanisms, miscoding can result in permanent DNA mutations leading to the development of cancer. Cells with damaged DNA are generally removed from tissue by apoptosis however, if there is a permanent mutation in a vital region of an oncogene or tumour suppressor gene it can cause oncogene activation or tumour suppressor gene deactivation. This leads to abnormal cellular proliferation or loss of normal growth control which can then lead to the development for example of lung cancer.[87,89] Besides initiating these processes cigarette smoke has also been identified as a tumour promoter.[87] Synergism between components in cigarette smoke can also increase the risk for lung cancer.

2.5 ANTIOXIDANTS

According to Halliwell and Gutteridge[92] an antioxidant is "any substance that when present at low concentrations compared with that of an oxidizable substrate significantly delays or inhibits oxidation of that substrate".[92] These include both enzymatic and non-enzymatic antioxidants. Antioxidants efficiently prevent the accumulation of oxidative elements thereby preventing or delaying the onset of diseases such as cancer, heart disease, COPD and aging.[93]

2.5.1 TYPES OF ANTIOXIDANTS

Antioxidants can be divided according to their origin, nature, chemical-physical properties, structure and their mechanism of action as shown in Table 2.1.

Table 2.1: Summary of antioxidant types [94]

Origin	Natural or synthetic
Nature	Enzymatic or non-enzymatic
Chemical-physical properties	Hydrophilic or hydrophobic
Structure	Polyphenols, flavonoids etc.
Mechanism	Preventative, chain breaking, etc

2.5.1.1 ENZYMATIC ANTIOXIDANT SYSTEMS

All eukaryotic organisms have effective enzymatic antioxidant defence systems and include the enzymes SOD, catalase GPx and GR.[95] Antioxidant enzymes provide protection by directly scavenging $O_2^{\bullet}$ radicals and H_2O_2 and converting them to less reactive species. Non-enzymatically molecular oxygen is converted to $O_2^{\bullet}$. SOD converts this $O_2^{\bullet}$ to H_2O_2. Even though H_2O_2 is not a radical, radicals can be formed from H_2O_2 by the Fenton reaction. Catalase neutralizes H_2O_2 forming $H_2O + O_2$, where O_2 can form $O_2^{\bullet}$. GPx neutralizes H_2O_2 by removing both hydrogen atoms from two GSH molecules which results in the formation of two H_2O molecules and one GSSG (oxidized GSH). GR then regenerates GSH from GSSG as shown in Figure 2.2.[96]

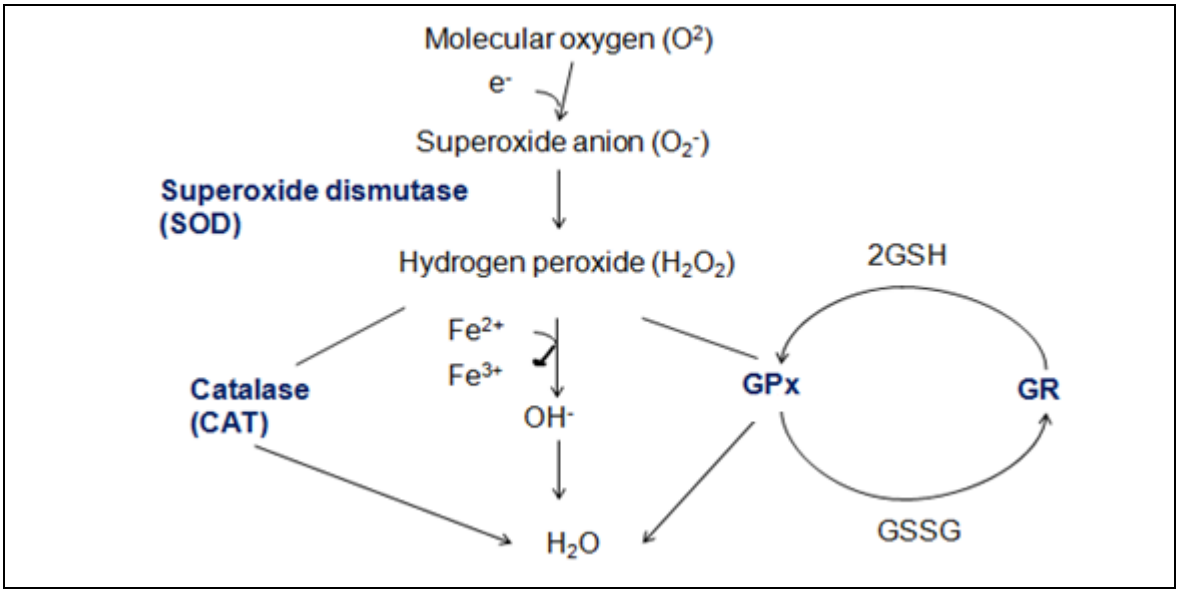


Figure 2.2: Mechanism showing how antioxidant enzymes reduce levels of the superoxide anion (Adapted from Mozaffaarieh[96], 2008).

2.5.1.2 NON-ENZYMATIC/CHEMICAL ANTIOXIDANTS

Non-enzymatic antioxidants are classified into two groups, namely hydrophilic and hydrophobic antioxidants. Hydrophilic antioxidants are able to dissolve into blood and cytosol and can react with free radicals.[93] These non-enzymatic antioxidants include antioxidant enzyme cofactors such as selenium and coenzyme Q10, oxidative enzyme inhibitors such as aspirin, transition metal chelators such as ethylenediaminetetraacetic acid (EDTA) and radical scavengers such as ascorbic acid and E.[97] Non enzymatic antioxidants have two different mechanisms of action. Firstly, these molecules can act directly by scavenging free radicals or secondly, by indirectly taking part in the regulation of enzyme activity.[94]

2.5.1.3 VITAMINS AS ANTIOXIDANTS

Besides their described physiological effects, several vitamins also have antioxidant activity. This discussion focused on vitamin E and C as the effects of these vitamins on metal induced oxidative damage was investigated.

Tocopherols and tocotrienols (TOC) are a class of vitamin E derivatives that are present in biological membranes. In membranes, TOCs have both antioxidant and non-antioxidant functions. The non-antioxidant function of these TOC's is to provide stability to the membranes and play an important role in the fluidity and permeability of membranes. As an antioxidant, these TOC's act as chain breaking components, which prevent the promotion of chain lipid autoxidation.[94]

Ascorbic acid (ascorbic acid) is a soluble micronutrient that is needed for multiple biological functions. It acts as a cofactor for several enzymes, and participates in post-translational hydroxylation of collagen. It is also plays an important role in the uptake of Fe, promoting absorption of Fe in the gastrointestinal tract (GIT). Ascorbic acid is also known for its reducing property which protects cellular components from oxidative damage. It has been shown to scavenge oxidising free radicals and other harmful oxygen-derived species. Ascorbic acid exhibits pro-oxidant effects associated with increased H_2O_2 formation which is responsible for its antibacterial and antiviral properties.[98]

2.5.1.4 DIETARY POLYPHENOLS AS ANTIOXIDANTS

Polyphenols are the most abundant dietary antioxidants.[99,100] Most polyphenols are secondary metabolites of plant origin and are present in polyphenol rich fruits, vegetables and beverages

such as wine and tea.[101] These molecules are reducing agents and play a role in protecting the body against oxidative stress and associated diseases such as neurodegenerative diseases, cardiovascular disease and cancer.[101]

Polyphenols are defined according to the structure of their carbon skeleton dividing them into different classes, ie, anthocyanins, proanthocyanidins, flavanols, flavones, flavanols, flavanones, isoflavones, hydroxybenzoic acids, hydroxycinnamic acids, stilbenes and lignans.[100]

Phenolic acids are found in large number of food sources. The most abundant phenolic acids are caffeic and ferulic acid. Caffeic acid is usually found in the form of esters and the most common form of this ester found in the diet is chlorogenic acid which is present in various fruits, vegetables and in coffee. Other forms of phenolic derivatives are the hydrolysable tannins. These are either gallic acid in gallotannins or other forms of phenolic acids which contain galloyl residues. These antioxidants can be found in berries, wine and brandy that have been aged in oak barrels. Finding gallic acid as a free molecule in the diet is rare as gallic acid usually occurs as structural components of condensed tannins.[100,101]

Flavonoids can be further divided into several classes and are found in a wide variety of vegetables and fruits (Table 2.2). Flavonoids as antioxidants are potent scavengers of radicals and are metal chelators. In addition, flavonoids have hypolipidemic, antithrombotic, and vasoprotective effects.[100-102]

Table 2.2: Summary of the flavonoid types and sources [100-102]

Type of flavonoid	Food source of flavonoid
Flavones	Red pepper, celery, parsley
Flavonols (quercetin)	Many fruits, vegetables and beverages including onions
Isoflavones	Soy-bean derived products
Anthocyanins	Red fruits such as cherries, plums, strawberries, grape, red and black currents, aubergine, rhubarb
Flavanols (catechins)	Abundant in green and black tea, red wine, chocolate, apples, beans
Proanthoctanidins	Apples, pears, grapes, wine, tea, chocolate
Flavanones	Citrus fruits

Flavonoids can protect the body against ROS. These molecules can act alone by directly scavenging free radicals or they can have an additive effect together with endogenous scavenging compounds.[103] Flavonoids can also reduce RNS formation as shown in the

reaction below, by directly scavenging the $O_2^{\bullet-}$ radical, thereby protecting cells and tissue against the effects of the highly reactive peroxynitrite radical ($ONOO^-$).[103]

NO (nitric oxide) + $O_2^{\bullet-}$ (superoxide) → $ONOO^-$ (peroxynitrite)

Flavonoids also inhibit xanthine oxidase, that catalyses the conversion of xanthine to uric acid with the formation of H_2O_2. [103]

During ischemia and inflammation, leukocytes which normally move freely, bind to the endothelial wall and become immobilised. This results in an increase in the levels of oxygen-derived free radicals, the release of other cytotoxic oxidants and inflammatory markers which cause cellular injury. Administration of oral flavonoids has been shown to decrease the number of immobilised leukocytes and consequently decrease free radical production and oxidative damage.[103] Flavonoids can chelate Fe and other metals thereby removing metal ions responsible for the production of free radicals from H_2O_2 via the Fenton reaction. The anti-inflammatory and antithrombogenic properties of flavonoids are due to the inhibition of arachidonic acid metabolism.[103]

2.6 PROTECTIVE EFFECT OF ANTIOXIDANTS AGAINST HEAVY METAL TOXICITY

Cessation of smoking is one of the most effective ways to reduce Pb, Cd, Cr and Al exposure. In addition, several studies have shown that antioxidants can protect against heavy metal induced oxidative damage. Examples of protective effects of antioxidants against specifically heavy metals Pb, Cd, Cr and Al will be described.

2.6.1 Phenolic acids

Phenolic acids are secondary metabolites that are widely spread throughout the plant kingdom. They are known for their various effects and uses in agricultural, biological, chemical and medical studies.[104] Phenolic acids have antioxidant, radical scavenging and cellular protective effects.[105]

2.6.2 Flavonoids

Flavonoids such as quercetin chelate Pb by forming coordination bonds with the Pb ions. An extract of garlic protects rats against Pb induced liver toxicity.[106] The antioxidant curcumin has both radical scavenging activity and metal chelating abilities.[106]

In an *in vitro* model, tea catechins were found to have protective effects against altered oxidative stress parameters and ROS formation caused by Pb exposure in PC12 cells (a cell line derived from a pheochromocytoma of the adrenal medulla of a rat). In addition, tea catechins were found to increase cell viability in the PC12 cells.[107] Polyphenols EGCG (catechin), quercetin and rutin protected mouse erythrocytes against Cr(VI) induced damage.[108] Tea catechins, especially those found in green tea have been reported to have neuroprotective properties. One of the mechanisms by which this occurs is likely the metal/antioxidant chelating characteristic of the catechin polyphenols against metals like Al, which have been associated with the pathogenesis of neurodegenerative diseases such as Alzheimer's disease.[109]

2.6.3 Antioxidant vitamins: Ascorbic acid, B and E

Deficiencies of antioxidant vitamins such as ascorbic acid, B_1 and B_6 have been associated with an enhanced sensitivity towards Cd and Pb toxicity. Vitamin supplementation has proved to be effective against Cd and Pb toxicity in both animals and humans.[110,111] The antioxidant, selenium was found to protect adult rat lungs against Al induced lung damage.[112] Vitamins B, C and E have been shown to chelate Pb thereby restoring the pro/antioxidant balance.[106] Vitamin E supplementation was also found to reduce Pb toxicity by inhibiting Pb absorption and this effect is possibly due to vitamin E chelation of Pb.[32]

El-Demerdash[113]*et al*. (2004) showed that vitamin E and β-carotene protected male rats exposed to Pb from lipid peroxidation, haematological changes and improved semen quality.[113] El-Demerdash[113] showed that vitamin E and selenium reduced lipid peroxidation and increased antioxidant enzyme activities in rats exposed to Al by reducing the levels of free radicals. Vitamin E was also found to reduce the effects of Al induced degeneration in rat testis.[114] Sugiyama[115]*et al*. (1991) reported that vitamin E protected hamster cells against cellular damage and mutations caused by sodium chromate exposure. The results of this study showed that vitamin E may be both antimutagenic and anticarcinogenic against the effects of Cr compounds.[115] In a cell culture based study it was shown that intracellular vitamin E prevented Cr mediated inhibition of GR (Figure 2.2) which would result in reduced GSH generation. Vitamin E and selenium have been shown to decrease Cr(VI) toxicity in broiler chicks.[116]

In animal studies, ascorbic acid was found to significantly reduce blood Pb levels.[19] In a cell culture study, intracellular ascorbic acid was found to reduce the toxicity and mutagenicity of

Cr. Ascorbic acid was also found to reduce Cr induced submandibular gland damage in rats.[117,118]

These *in vitro* and *in vivo* studies indicate that antioxidants protect against heavy metal induced oxidative damage.

2.6.4 Additive and synergistic effects between antioxidants

Vitamin E, ascorbic acid and selenium in combination inhibited rat testicular damage induced by Cd.[119] Exogenous antioxidant supplementation including ascorbic acid, vitamin E, β-carotene, Coenzyme Q10, and green tea replenished endogenous antioxidant supply thereby reducing oxidative stress and free radical formation[120].[120]

It has recently been hypothesized that oxidative stress is one of the main participants in the pathogenesis of hypertension. A combination supplement containing ascorbic acid, vitamin E, β-carotene and zinc resulted in a significant decrease in systolic blood pressure but a non-significant reduction in diastolic blood pressure.[121,122]

Synergistic beneficial effects are related to the specific bioactivity of each antioxidant related to radical scavenging, chelating ability and targeting of specific enzymes such as xanthine oxidase and antioxidant pathways such as the GSH pathway.

2.7 CLINCAL STUDIES: CHRONIC DISEASES, SMOKING AND ANTIOXIDANTS

Cigarette smoke contains a variety of toxic substances that causes cellular and tissue damage, and one of the mechanisms whereby the heavy metals found in cigarettes mediate toxicity is via the formation of ROS contributing significantly to diseases such as COPD and lung cancer. Since ROS and oxidative stress play a major role in the genesis of these diseases, antioxidants should aid in their prevention and treatment. Although the use of antioxidants in the prevention and treatment of COPD is limited, antioxidants can play an important role in reducing oxidative damage and inflammation associated with COPD.[123]

Vitamin E has been found to have a beneficial effect on lung function, thereby delaying or preventing the development of COPD.[124] N-acetlycysteine (NAC) a thiol antioxidant, which is also a precursor for GSH was found to reduce pulmonary emphysema in rats.[125] It has also been reported that through the reduction of O_2^- to H_2O_2, the antioxidant enzyme SOD can decrease damage to the lung caused by emphysema.[126]

Certain cruciferous plants such as broccoli are being evaluated for the treatment of COPD, as the antioxidants found in these plants positively impacts on endogenous redox processes. These plants contain a range of molecules including natural antioxidants, folate, ascorbic acid and β-carotene.[123] A pilot study conducted on the effect that broccoli has on male smokers showed that broccoli caused plasma antioxidants to be upregulated, it influenced GSH activity and increased cellular defences against oxidative stress.[127]

Much debate has arisen about whether antioxidant supplementation is beneficial to cancer patients and whether it alters the efficacy of chemotherapy.[128] A number of studies have been conducted where antioxidant treatment has been incorporated into treatment for cancer patients undergoing chemotherapy and radiation treatment.[129-131] Preliminary evidence is limited due to sample size and quality, but does suggest that addition of antioxidants to the chemotherapy regime is safe and that certain antioxidant supplements may reduce adverse effects of chemotherapeutic and radiation treatment like mucositis/stomatitis, neurotoxicity and weight loss, resulting in improved quality of life.[128-131] A reduction in these side effects associated with toxicity may result in more patients completing their chemotherapy treatment. Besides reduced toxic effects, antioxidant supplementation may increase survival times and/or increased tumour response to therapy.[128]

Antioxidant treatment intervention studies in COPD and lung cancer is not well researched as the effect is often difficult to quantify, however indications are that antioxidant supplementation does reduce the amount of oxidative stress and has shown a decrease in toxicity.[132] Further research is required to identify the best antioxidant combinations related to specific targets, mechanisms as well as the measurement of endpoints related to antioxidant effects.

2.8 AIM AND OBJECTIVES

The aims of this exploratory study were to investigate the oxidative toxic effects of Pb, Cd, Cr and Al, found in cigarettes and to further investigate how dietary antioxidants can protect against this induced oxidative damage.

In the first part of the study the objectives were:

1. To determine the concentration of Pb, Cd, Cr and Al that causes 50% human erythrocyte haemolysis.
2. To compare the change in morphology of induced by Pb, Cd, Cr and Al, compared to that induced by a known oxidant AAPH.

3. To determine if Pb, Cd, Cr and Al can catalyse the Fenton reaction resulting in the formation of hydroxyl radicals.
4. To determine in *ex vivo* erythrocytes if Pb, Cd, Cr and Al induces ROS.
5. To determine if Pb, Cd, Cr and Al can bind GSH.
6. To determine in *ex vivo* erythrocytes if Pb, Cd, Cr and Al reduces GSH levels.

In the second part of the study the objectives were:

7. To determine whether dietary polyphenolic acids, flavonoids and vitamin derivatives, catechin, gallic acid and Trolox protects against Pb, Cd, Cr and Al induced haemolysis.
8. To determine if phenolic acids, flavonoids and antioxidant vitamins inhibit the Fenton reaction catalysed by Pb, Cd, Cr and Al.
9. To determine whether dietary polyphenolic acids, flavonoids and vitamin derivatives, catechin, gallic acid and Trolox protects against Pb, Cd, Cr and Al induced oxidative damage in erythrocytes.
10. To determine if polyphenolic acids, flavonoids and antioxidant vitamins inhibit ROS formation by binding metals.
11. To determine if polyphenolic acids, flavonoids and antioxidant vitamins inhibit metal/GSH interactions.
12. To determine if antioxidants binding to Pb, Cd, Cr and Al alters the antioxidant activity of antioxidants.
13. To determine whether dietary polyphenolic acids, flavonoids and vitamin derivatives, catechin, gallic acid and Trolox protects against Pb, Cd, Cr and Al induced changes in GSH levels.

CHAPTER 3: MATERIALS AND METHODS

All methodologies required to achieve the objectives listed in Section 2.8 are contained in sections 3.1 and 3.2 and a summary of the experimental procedures is presented in Figure 3.1. All research was conducted in the research facilities of the Departments of Anatomy and Pharmacology of the Faculty of Health Sciences and the Unit for Microscopy and Microanalysis, University of Pretoria.

3.1 MATERIALS

3.1.1 REAGENTS, EQUIPMENT AND DISPOSABLE PLASTICWARE

Reagents, 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH), catechin, Trolox, gallic acid, ascorbic acid, caffeic acid, quercetin, Fe(II) sulphate hydrate, fluorescein, 5,5'-dithiobis–(2-nitrobenzoic acid) (DTNB), Al chloride ($AlCl_3$), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), were obtained from the Sigma-Aldrich Company, Atlasville South Africa (SA). Iso PBS (isotonic phosphate buffered saline) (0.137M NaCl; 3M KCl; 1.9 M NaH_2PO_4. $2H_2O$; 8.1 M Na_2HPO_4) were obtained from Merck Chemicals, Modderfontein SA. The metals lead acetate ($Pb(C_2H_3O_2)_2$), cadmium sulphate ($CdSO_4$), chrome potassium sulphate ($CrK(SO_4)_2$) and aluminium ammonium sulphate ($AlNH_4(SO_4)_2$) were also obtained from Merck Chemicals, Modderfontein SA.

3.1.2 EQUIPMENT

Equipment that was used includes: BioTek plate reader purchased from Analytical and Diagnostic Products (ADP) Johannesburg, SA. Hermle Z300 centrifuge, Crison GLP 21 pH meter and Eppendorf pipettes from Eppendorf AG Hamburg, Germany were all supplied by the Scientific Laboratory Equipment Company (LASEC), Cape Town, SA.

3.1.3 GLASSWARE AND PLASTICWARE

All glassware and disposable plasticware including 96 well plates, 600ml Eppendorf tubes, and pipette tips (10, 25, 100, 200, and 1000µl) were from Greiner Bio-one supplied by LASEC, Cape Town, SA and NUNC^{TM} supplied by AEC-Amersham, Johannesburg, SA.

3.1.4 SAMPLE COLLECTION

Blood samples were obtained from ten healthy, non-smoking, consenting volunteers with ethical approval, ethics number 268/2015. Approval was obtained from the Research Ethics Committee, Faculty of Health Sciences, University of Pretoria.

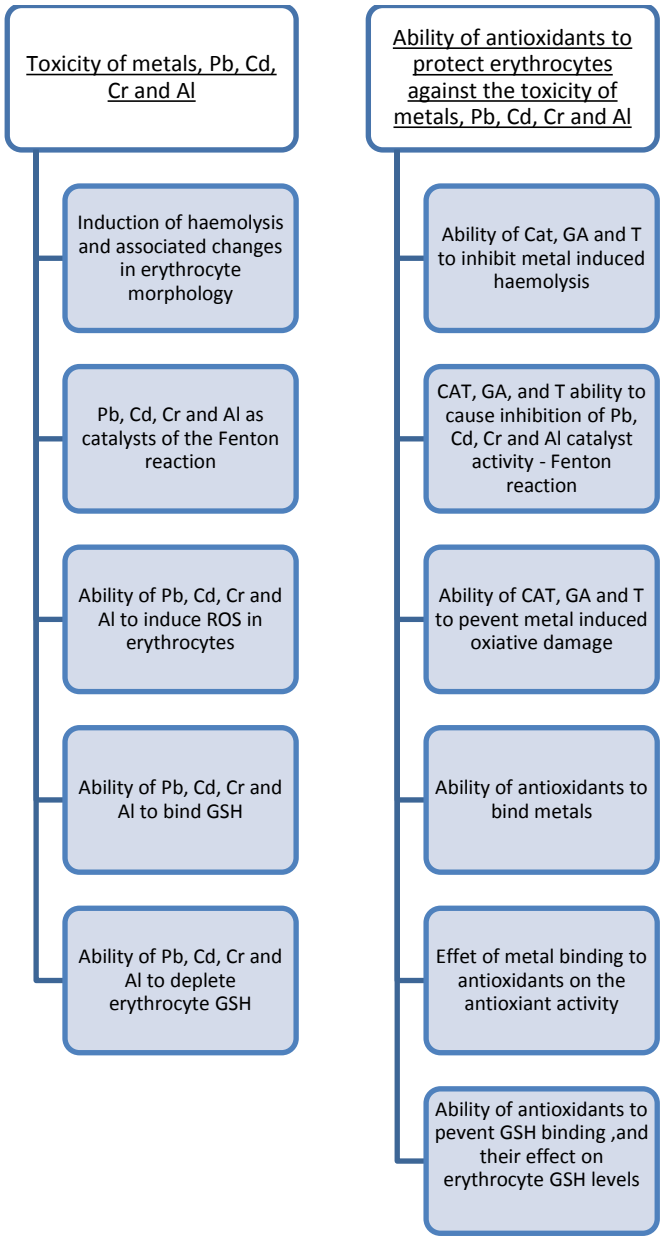


Figure 3.1: A schematic representation of the experimental design.

3.2 METHODS

3.2.1 HAEMOLYTIC, OXIDATIVE EFFECTS RELATED TO RADICAL FORMATION AND GSH BINDING/DEPLETION

3.2.1.1 HEAMOLYTIC EFFECTS OF Pb, Cd, Cr AND Al

The haemolysis assay was used to determine the concentrations at which Pb, Cd, Cr and Al caused erythrocyte haemolysis. A blood sample was collected from a consenting donor, thereafter the erythrocytes were collected by centrifugation at 5000xg in the laboratory. A 5% solution was then made by diluting the erythrocytes in an isotonic phosphate buffered solution (IsoPBS) (0.137M NaCl, 0.003M KCl, 0.0019M $NaH_2PO_4.2H_2O$, 0.0081M Na_2HPO_4 containing 0.005M of glucose) and were either used immediately or stored at 4°C for a maximum of five days. For each assay done in triplicate a 100µl sample was placed in a 600µl microcentrifuge tube. The positive control (H_{100}) was prepared by adding 40µl of a 2% sodium dodecyl sulfate (SDS) (an ionic detergent that causes erythrocyte lysis) solution to the 5% erythrocyte suspension. The negative control (H_0) contained 40µl IsoPBS solution added to the 5% erythrocyte suspension.

For all metals a 1.0M stock solution was prepared and from these, working solutions at the respective concentrations were further prepared. A volume of 40µl of each working solution was added to a 100µl volume of the erythrocyte suspension in 600µl microcentrifuge tubes.

For Pb acetate (Pb(II) referred to as Pb), from the stock solution, 25, 35 and a 45mM working solutions in dH_2O were prepared. The final concentrations were 7, 10 and 13mM. For Cd sulphate (Cd(II) referred to as Cd) from the stock solution 20, 40 and 60mM working solutions in dH_2O were prepared. The final concentrations were 6, 12 and 18mM. Likewise, for Cr potassium sulphate (Cr(III) referred to as Cr) from the stock solution, 85, 95 and a 105mM working solution in dH_2O were prepared. The final concentrations were 25, 27 and 30mM. Finally, for Al ammonium sulphate (Al(III) referred to as Al) working solutions of 180, 200 and 220mM working solutions in dH_2O were prepared. The final concentrations were 57, 60 and 63mM.

All tubes were gently mixed and were incubated at 37°C for 16 hrs. After this period of incubation, the erythrocytes were re-suspended and thereafter centrifuged at 3500g for 2

minutes (min). A 50µl volume of the supernatant was placed into the wells of a 96 well plate and the absorbance was measured at a wavelength of 540nm using a BioTek plate reader. The percentage haemolysis was calculated as follows:

$$\textbf{Percentage haeomolysis} = H_{sample} - H_0 / H_{100} - H_0 \times 100$$

3.2.1.2 EFFECTS OF AAPH AND METALS ON ERYTHROCYTE MORPHOLOGY

Scanning electron microscopy (SEM) is used to evaluate the effect of molecules such as metals on the surface morphological features of cells such as erythrocytes. Toxicity such as oxidative damage causes changes in the plasma membrane structure resulting in increased lipid peroxidation, loss of trans-membrane asymmetry, increased permeability and erythrocyte membrane blebbing.[133] The extent of oxidative damage is dependent on the concentration of the oxidant and incubation time.

In order to identify exposure time related changes to erythrocyte morphology, erythrocytes were exposed to AAPH and at specific time intervals and the morphology of the erythrocytes was then examined. In order to achieve this, the effect of 40µl of a freshly prepared 300mM AAPH solution (final concentration 85.7mM) on erythrocyte membrane morphology was evaluated at 1, 2, 6, 8 and 16 hrs. The typical morphological features were defined as described by Gyawali *et al*. 2015.[134] This experiment allowed for the description of the sequence of events that leads to haemolysis.

In order to evaluate the effects of the heavy metals the highest concentration in Figure 4.1(filled bar) was used, and these concentrations were for Pb 13mM; Cd 18mM; Cr 27mM and Al 63mM. The experimental conditions were as described in Section 3.2.1.1 and erythrocyte morphology was evaluated after 16 hrs of incubation.

For SEM, all samples were processed as follows: after incubation, 100µl of fixative (2.5% glutaraldehyde, 2% formalin in 0.1M phosphate buffer, pH 7.4) was added to the erythrocyte suspension. The microcentrifuge tube was inverted 3 times and the erythrocytes were then fixed for 1 hr. The erythrocytes were allowed to settle by gravity. A 100µl volume of PBS was added to the erythrocyte suspension in the 600µl microcentrifuge tubes. It was left for 5 min, and then removed. This was repeated three times per tube. 100µl osmium tetroxide was added to the erythrocytes and left to stand for 30 min, after which the erythrocytes were rinsed 3 times with PBS. The erythrocytes were then dehydrated through ethanol in the following ascending concentrations for 5 min each of 30%, 50%, 70% and 90 and finally 100% ethanol, three times, for 7 min each. The erythrocytes were dried by critical point drying and chemically

treated with hexamethyldisilazane (HMDS) under a fume hood for 30 min. The HMDS was removed by centrifugation and fresh HMDS was added to the centrifuge tubes. Two drops of the cell suspension were dropped onto a cover slip and left to dry. The erythrocytes were coated with carbon evaporation and viewed using Carl Zeiss Ultra Plus Field emission SEM.[135]

3.2.2 OXIDATIVE EFFECTS OF Pb, Cd, Cr AND Al

Oxidative damage can be the result of the formation of reactive hydroxyl, oxygen and nitrogen species and/or the depletion of the cellular radical scavenging components such as GSH.

3.2.2.1 PB, CD, CR AND Al AS CATALYSTS OF THE FENTON REACTION

In the Fenton reaction, metals such as Fe catalyse the formation of $OH^{\bullet}$ radicals from H_2O_2. In the hydroxyl radical averting capacity (HORAC) assay, the ability of antioxidants to prevent $OH^{\bullet}$ mediated quenching of fluorescein is measured. Although generally the Fenton reaction involves Fe, in the HORAC assay, Co as an oxidising agent was proven to be more effective and a better catalyst of $OH^{\bullet}$ formation.[136-138] Co was effective at low concentrations and effectively catalysed the formation of $OH^{\bullet}$ at a high pH via the reaction given below.[138]

$$Co(II) + H_2O_2 \rightarrow Co(III) + OH^{\bullet} + OH^{-}$$ [139]

In order to determine the ability of Pb, Cd, Cr and Al to catalyse the formation of $OH^{\bullet}$, 10µl of each metal solution (45mM Pb, 60mM Cd, 105mM Cr and 220mM Al) was added to separate wells in a 96 well plate. A 180µl volume of a 0.01mM fluorescein solution was added to the wells. After 2 min of incubation at 37ºC, 20µl H_2O_2 was added to each well. The final concentrations of each metal were 2.14mM Pb, 2.85mM Cd, 5.00mM Cr and 10.47mM Al. The change in fluorescence was read at an excitation wavelength of 485nm and emission at 520nm, twice every min for 30 cycles. The negative control (0% ROS production) contained 10µl PBS added to 180µl fluorescein and 20µl H_2O_2. Co was used as a positive control and the final concentration of Co was 0.46mM.[137]

3.2.2.2 ABILITY OF METALS TO INDUCE OXIDATIVE DAMAGE IN ERYTHROCYTES

The DCFH-DA assay is an assay used to detect oxidative damage caused by ROS and RNS.[140] Erythrocytes exposed to AAPH will form ROS and the intracellular levels can be measured using the DCFH-DA assay. The DCFH-DA assay is an assay widely used to detect

oxidative damage caused by ROS and RNS. In this assay, the DCFH-DA diffuses into the erythrocytes where it is hydrolysed to DCFH by intracellular esterases. The peroxyl radicals generated from the oxidant (for example AAPH), oxidises the DCFH to its fluorescent derivative DCF. Thus, an increase in fluorescence indicates oxidative activity.[141] If oxidative damage occurs the non-fluorescent DCFH-DA forms the fluorescent diacetate (FDA).

In order to a volume of 225µl of the erythrocyte suspension which was prepared as described in section 3.2.1.1, 225µl of a 75mM DCFH-DA solution was added. Tubes were incubated at 37°C for 1 hr and the erythrocytes were then collected by centrifugation at 3500g for 2 min. The erythrocytes were washed twice then re-suspended in 450µl of IsoPBS. In order to a 75µl volume of the erythrocytes 75µl of a 45mM Pb, 60mM Cd, 105mM Cr and 220mM Al solution was added. The final concentration of each metal was 35.5mM Pb, 30mM Cd, 52.5mM Cr and 110mM Al. As a positive control, AAPH was used and 75µl of a 20mM AAPH, final concentration 10mM was added. For the negative control 75µl volume of IsoPBS solution was added. The change in fluorescence was measured at 0 to 5 min following the addition of the metals. An excitation wavelength of 485nm and an emission wavelength of 520nm were used. The gradient from 0-20 min (cycle 1-9) was determined.

3.2.2.3 GSH BINDING AND EFFECTS OF METALS ON ERYTHOCYTE GSH LEVELS

The reduced form of GSH is the principle free thiol found in almost all living cells and is a part of numerous biological processes which includes the detoxification of xenobiotics, the removal of hydroperoxides, and maintaining the oxidation state of protein sulfhydryls. Glutathione is mostly found in cells in its reduced state as GSH (between 90-95% of total glutathione) and the remainder in the oxidised state, GSSG. Intracellular levels of reduced GSH levels are good indicators of the overall cellular health and wellbeing as well as the ability of a cell to cope with toxins and other stressors such as oxidative stress. Metals can directly bind GSH or inhibit GSH recycling dependent enzymes and this can result in the accumulation of GSH or depletion of GSH depending on the inhibited enzyme (Figure 2.2).

3.2.2.4 DETERMINATION OF METAL-GSH BINDING

GSH is a cysteine containing tripeptide and in biological systems the amount of free cysteine can be quantified in protein using DTNB also known as Ellman's Reagent. The ability of Pb,

Cd, Cr and Al to bind to GSH was determined. Binding of these metals to GSH would decrease the amount of free GSH that can bind DTNB.

A 1mM GSH solution was prepared in a PBS solution (0.2M Na_2HPO_4, 0.2M $NaH_2PO_4.H_2O$, 0.15M NaCl, pH 8). A pH of 8 is necessary for GSH to bind DTNB. In order to prepare a 10mM DNTB stock solution, 3.96mg DTNB powder was first dissolved in 100µl methanol (MeOH), sonicated until dissolved and was then added to 900µl of PBS. A 0.01mM working solution was then prepared from the stock solution. In addition to 50µl of the GSH solution, 50µl of the metal solutions were added, mixed well and then 50µl DNTB was added. The volumes of all solutions were adjusted to a final volume of 200µl with the addition of 50µl H_2O. After an incubation period of 5 min the absorbance was measured at 405nm using a BioTek plate reader. In each well the final concentration of GSH was 0.25mM. The final concentrations of Pb, Cd, Cr and Al were 13, 18, 30 and 63mM respectively and were in excess of GSH. All results were expressed as a percentage of the control, GSH alone with no metals added and were calculated as follows:

$$\textbf{Percentage } GSH_{unbound} = Abs_{sample} - Abs_{metal\ blank} / Abs_{GSH\ alone} - Abs_{PBS\ blank}$$

3.2.2.5 EFFECTS OF METALS ON ERYTHOCYTE GSH LEVELS

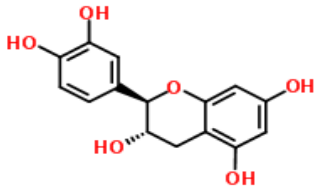
In order to determine the effect of each metal on erythrocyte GSH levels an erythrocyte suspension was prepared as for the haemolysis assay in 3.2.1.1. A 4µl volume of three concentrations of each metal was added to 100µl of the erythrocyte suspension in 600µl microcentrifuge tubes. These were 25, 35 and 45mM solutions for Pb with final concentrations of 7, 10 and 13mM. For Cd this was 20, 40 and 60mM and the final concentrations in solution were 6, 12 and 18mM. Likewise, for Cr 85, 95 and 105mM working solutions were used and the final Cr concentrations were 25, 27 and 30mM. The concentrations of the working solutions for Al were 180, 200 and 220mM and the final concentrations were 57, 60 and 63mM. For the control a 40µl volume of PBS was added instead of metals.

The erythrocytes were then incubated at 37°C for 16 hrs. After incubation the cells were haemolysed by adding 100µl of a 2% SDS solution to each tube. Thereafter the erythrocytes were vortexed for 1 min to ensure complete haemolysis before a 100X dilution was prepared. A 50µl volume of the diluted haemolysed erythrocytes was pipetted into the wells of a 96 well plate. A volume of 100µl DTNB prepared as described above was added and the absorbance was measured at 405nm, 5 min after adding the DTNB.

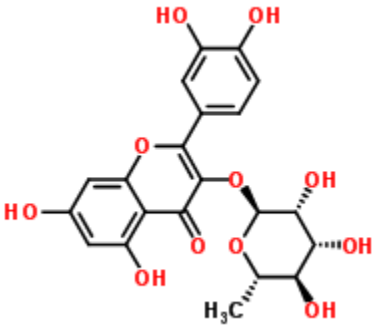
3.2.3 EFFECTS OF POLYPHENOLICS, FLAVONOIDS AND VITAMIN DERIVATIVES ON METAL INDUCED OXIDATIVE DAMAGE AND GSH LEVELS

Metal catalysed oxidative damage can be reduced by antioxidants. These molecules act by directly binding the metal, preventing the depletion of GSH and/or scavenging the formed radicals. The ability of several antioxidants (Figure 3.2) to prevent haemolysis, to scavenge radicals, to prevent binding to GSH and to prevent erythrocyte depletion was evaluated. Finally the effect of metals on the antioxidant activity of the polyphenols and antioxidant enzymes was determined.

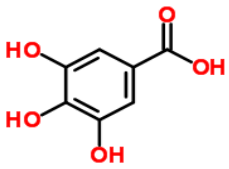
Catechin



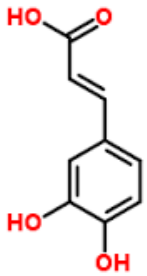
Quercetin



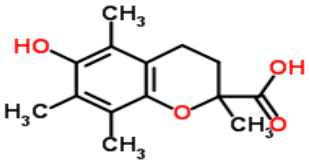
Gallic acid



Caffeic acid



Trolox



Ascorbic acid

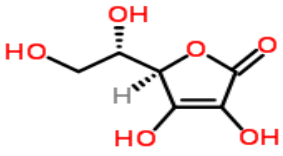


Figure 3.2: Chemical structures of the polyphenols and antioxidant vitamins used in this study. Structures from Chem-Spider.[142]

3.2.3.1 ABILITY OF ANTIOXIDANTS TO PREVENT ERYTHROCYTE HAEMOLYSIS

The ability of antioxidants; catechin, gallic acid and Trolox to inhibit Pb, Cd, Cr and Al induced haemolysis was determined. Erythrocyte suspensions were prepared as described in section 3.2.1.1. Metal concentrations that induce H_{50} were used (Table 4.1) and the effect of increasing concentrations of antioxidants was then evaluated. The final concentration of each metal was 13mM Pb, 18mM Cd, 30mM Cr and 63mM Al. The working concentration for each antioxidant evaluated was 1mM with the final concentration being 0.2mM. For the positive control, 40µl volume of a 2% SDS solution was used and for the negative control a 40µl volume of IsoPBS solution was used.

All tubes were gently mixed and were incubated at 37°C for 16 hrs, after which the erythrocytes were collected by centrifugation at 3500xg for 2 min. A 50µl volume of the supernatant was placed into the wells of a 96 well plate and the absorbance was measured at 540nm and the percentage haemolysis was calculated as follows:

$$\textbf{Percentage haemolysis} = H_{sample} - H_0/H_{100} - H_0 \times 100$$

The percentage haemolysis calculated for the metal antioxidant combinations was then compared to the percentage haemolysis caused by the metals alone. Statistical difference ($p \leq 0.001$) indicated a protective effect and was calculated using One-Way Analysis of Variance (ANOVA) with a confidence level of 95%.

3.2.3.2 ABILITY OF ANTIOXIDANTS TO INHIBIT THE FENTON REACTION

All metal and fluorescein solutions were prepared as described in Section 3.2.2.2. A volume of 50µl of 1mM catechin, quercetin, gallic acid, caffeic acid, Trolox and ascorbic acid was added. The final concentration of each antioxidant was 0.025mM. The change in fluorescence was measured at an excitation wavelength of 485nm and emission at 520nm, twice every min for 30 cycles. The negative control (0% ROS production) contained 10µl antioxidant added to 180µl fluorescein and 20µl H_2O_2.

3.2.3.3 ABILITY OF ANTIOXIDANTS TO PREVENT ERYTHROCYTE OXIDATIVE DAMAGE

The DCFH-DA assay as described in Section 3.2.2.2 was used. A third of the reaction volume of reaction mixture was replaced with 75µl of 1mM catechin, gallic acid or Trolox. The final concentration of each antioxidant was 0.3mM. As described in Section 3.3.2.1 the change in fluorescence was measured at an excitation and emission wavelength of 485nm and 520nm respectively. The effect of antioxidants on the measured levels of percentage oxidative damage for each metal was calculated. Statically significant differences in the measured levels of oxidative damage indicated a possible protective effect.

3.2.3.4 ABILITY OF ANTIOXIDANTS TO INHIBIT THE FENTON REACTION BY BINDING METALS

The traditional $AlCl_3$ method used for the quantification of flavonoids involves the formation of acid stable complexes with the C-4 keto group and either the C-3 or the C-5 hydroxyl group of flavones and flavonols. Flavonoids that bind Al, would reduce the ability of Al to take part in the Fenton reaction. Likewise using the same strategy was possible to determine if Pb, Cd and Cr also bind flavonoids thereby reducing the toxic effects of these metals. The ability of catechin, quercetin, gallic acid, caffeic acid, Trolox and ascorbic acid to bind metals was also determined.

The original method used for the determination of the total flavonoid content of samples is as follows: A 10µl volume of increasing concentrations of flavonoids or antioxidant containing mixtures was added to the wells of a 96 well plate. A volume of 30µl of a 2.5% solution of sodium nitrite is then added to the samples in the plate followed by 25µl of a 2.5% $AlCl_3$ solution after which 100µl of a 2% sodium hydroxide solution was added. The absorbance was measured at 405nm.[140]

In this study the above method was slightly adapted. The 2.5% $AlCl_3$ solution was replaced with a 116mM Pb, 157mM Cd, 270mM Cr and a 566mM Al solution. In this assay the final metal concentrations were 13mM, 18mM, 30mM and 63mM respectively. A concentration series of each antioxidant was used and this was 0, 0.16, 0.33, 0.67, 0.8 and 1mM.

3.2.3.5 EFFECT OF METAL BINDING TO ANTIOXIDANTS ON THE ANTIOXIDANT ACTIVITY

Several assays can be used to determine antioxidant activity. Two main types of assays are the electron transfer and the hydrogen atom transfer assays. The TEAC assay which is an electron transfer assay measures the ability of an antioxidant to quench $ABTS^{\cdot+}$ which is bluish-green chromophore. Activity is compared to the antioxidant activity of Trolox which is a water-soluble vitamin E analogue. The addition of heavy metals may cause the flavonoids to bind the metals, thereby reducing the measured antioxidant activity of the flavonoids. When antioxidants are added to the radical cation it is reduced to ABTS. This results in a decolourization of the dark green $ABTS^{\cdot+}$ radical.[143]

A concentration of 7mM ABTS was dissolved in PBS and then 2.45mM potassium persulfate was added. The mixture was then left to stand at room temperature, in the dark for 12 hrs. This produced a dark bluish-green solution. A 1ml volume of this stock solution was diluted in a volume of 29ml of PBS to produce the working solution.

A 290µl volume of the ABTS working solution was then added to 10µl of 45, 60, 105 and 220mM solutions of Pb, Cd, Cr and Al respectively and the final concentrations were 22.5, 30, 52.5 and 110mM respectively. Then 10µl of 1mM solutions of catechin, quercetin, gallic acid, caffeic acid, Trolox and ascorbic acid was added. The final concentration for each antioxidant used in this assay was 0.33mM. Controls were ATBS without metals and antioxidants as well as ATBS with metals. The absorbance was measured at a wavelength of 630nm.[97]

3.2.3.6 ABILITY OF ANTIOXIDANTS TO INHIBIT METAL - GSH BINDING

GSH solutions were prepared as described in Section 3.2.2.4. Instead of the addition of 50µl H_2O, 50µl of a 1mM solution of catechin, gallic acid or Trolox was added prior to the addition of 50µl DTNB solution. The final concentration of GSH was 0.25mM and the metals were 13, 18, 30 and 63mM. The final concentration of each antioxidant was 0.25mM. The absorbance was measured at 405nm and all results were expressed as percentage of control, GSH alone with no metals added and was calculated using the following equation. Samples containing antioxidants were compared to samples containing metals and GSH.

$$\textbf{Percentage } GSH_{unbound} = Abs_{sample} - Abs_{metal\ blank} / Abs_{GSH\ alone} - Abs_{PBS\ blank}$$

3.2.3.7 EFFECT OF ANTIOXIDANTS ON ERYTHROCYTE GSH LEVELS

Erythrocyte suspensions were prepared as described in Section 3.2.1.1. Following the addition of 40µl of the metal solutions (Section 3.2.2.5), 40µl of 1mM catechin, gallic acid or Trolox was then added to the erythrocytes. After 16 hrs incubation at 37ºC GSH levels were quantified as described in section 3.2.2.4.

CHAPTER 4: RESULTS AND DISCUSSION

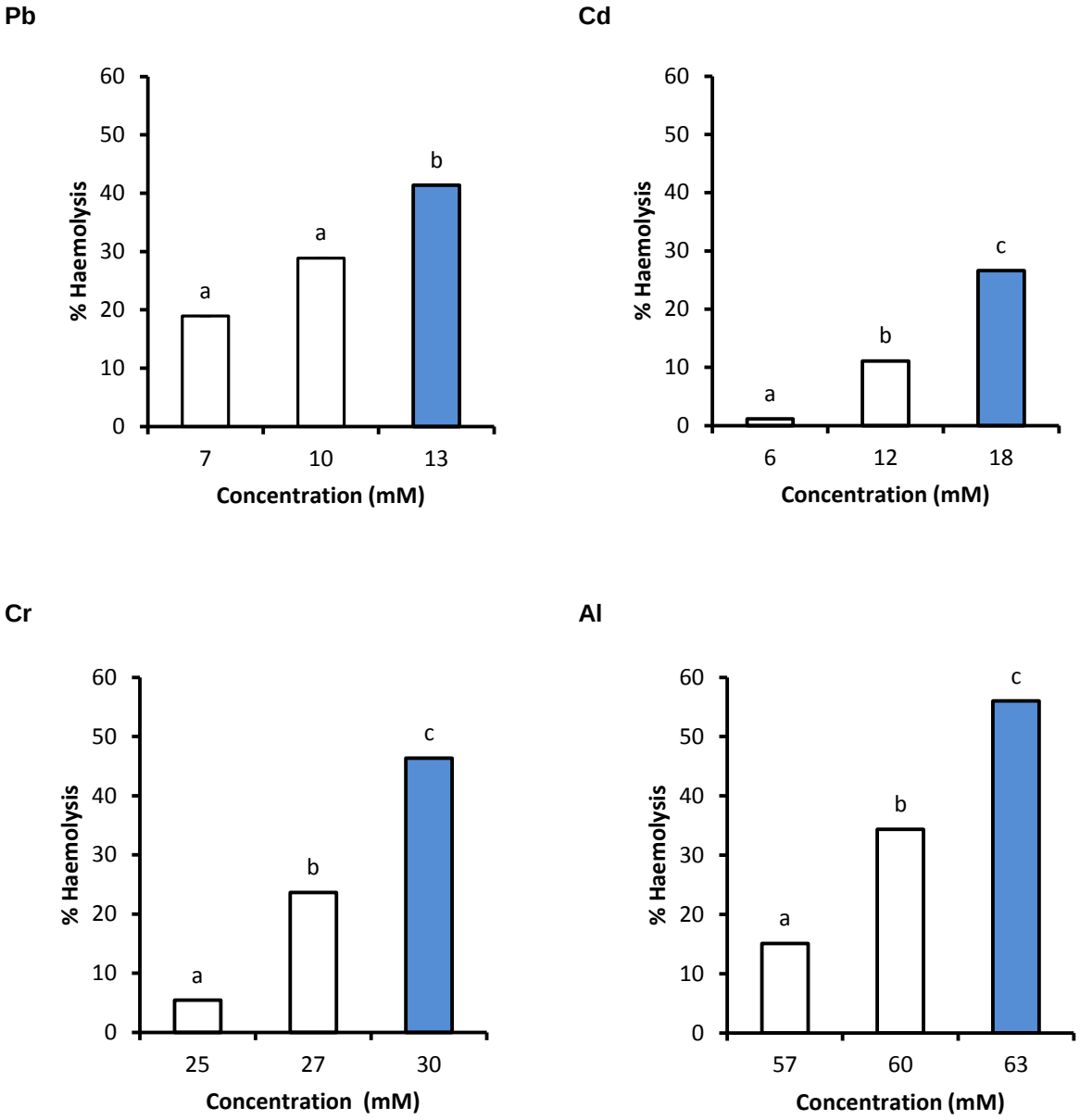
A: HEAVY METAL TOXICITY

Smoking contributes to 58% of lung cancer deaths, 37% of COPD deaths, 20% of TB deaths and 23% of vascular related deaths in South Africa. Approximately 8% of all deaths in South Africa (more than 20 000 deaths per year) are caused by smoking.[1] Cigarette smoke contributes to several preventable diseases worldwide which include COPD, lung cancer and IHD.[144] Cigarette smoke is a complex mixture of molecules which includes toxic hydrocarbons, gases as well as metals. In this study, the oxidative effects of metals Pb, Cd, Cr and Al that are found in cigarette smoke were evaluated.

4.1. CONCENTRATIONS OF PB, CD, CR AND AL THAT CAUSES SIGNIFICANT LEVELS OF HAEMOLYSIS IN HUMAN ERYTHROCYES

Erythrocytes contain a lipid bi-layer which surrounds and protects the cell. Once this lipid bi-layer is damaged haemolysis occurs and Hb escapes.[129] Quantification of the released Hb from erythrocytes is the basis of the haemolysis assay used to evaluate toxicity such as that which occurs with oxidative stress. The haemolysis assay measures the percentage haemolysis relative to a positive control that causes complete haemolysis (100%) and a negative control where no haemolysis occurs. Erythrocytes are exposed to a range of serial dilutions of a specific drug or chemical that is being tested.[129]

In order to determine the concentration of Pb, Cd, Cr and Al that causes haemolysis erythrocytes were exposed for 16 hrs to increasing concentrations of each metal. For each metal a dosage effect was observed across a narrow concentration range and this was for Pb (7-13 mM), Cd (6-18 mM), Cr (25-30 mM) and Al (57-63 mM) (Figure 4.1). The line equation of each graph was determined and from this the concentration of each metal that causes 50% haemolysis (H50%) was calculated (Table 4.1).



<u>Figure 4.1:</u> Ability of heavy metals Pb (7-13mM), Cd (6-8mM), Cr (25-30mM) and Al (57-63mM) to cause erythrocyte haemolysis at an exposure time of 16 hrs. Data is an average of three experiments with three data points each. Standard error of the mean < 0.02%. Means with different letters are significantly different, p≤0.001. The filled bar is the concetration for each metal that was further used in the present study.

Table 4.1: Line equations of graphs (Figure 4.1), curve fit (R^2) and the calculated concentrations causing haemolysis (Calculated H50%)

Metal	Equation	R^2	Calculated H50% (mM)
Pb	y = 3.1213x - 1.113	0.989	16.00
Cd	y = 1.4977x - 3.754	0.886	33.38
Cr	y = 1.0794x - 3.269	0.508	47.83
Al	y = 0.6270x - 1.858	0.606	81.26

The order of toxicity was Pb > Cd >Cr > Al. Pb was 2.06, 2.93 and 5.06 times more toxic than Cd, Cr and Al respectively.

Pb has been described to cause changes in the integrity of membranes, as well as changes in their permeability and function and possibly an increase in susceptibility to lipid peroxidation.[32,37] Lawton and Donaldson[145] reported that Pb has a destabilizing effect on cellular membranes. In erythrocytes, Pb decreased the fluidity of the cell membrane and increases the rate of erythrocyte haemolysis. This change in fluidity was the result of ROS formation that caused peroxidation of the lipids present in the outer membrane of the erythrocyte.[145,146] In patients, Pb exposure causes hypochromic or normochromic anaemia which is a result of ROS generation, which leads to membrane peroxidation and consequently erythrocyte haemolysis.[21,32] The blood erythrocyte counts, Hb levels, haematocrits and mean corpuscular volumes of workers exposed to Pb was lower than non-exposed workers.[147]

Kiyoatake[148] *et al*. (2015) examined the interactions of different metals on bio-membranes of human erythrocytes. In this study erythrocytes exposed to heavy metals were subjected to a pressure of 200MPa. Changes in membrane integrity due to Hg and Cd exposure increased cellular susceptibility to pressure induced haemolysis.[148]

Human erythrocytes were exposed to 0 – 160µM Cr(VI) and only after 48 hrs was haemolysis observed.[149] In the present study at a higher concentration of 25mM Cr(III) erythrocyte haemolysis was observed after 24 hrs.

Few studies are available on the effects of Al on human erythrocytes. The effects of macro-sized Al oxide (Al_2O_3) nanoparticles on rat, rabbit and human erythrocytes have been investigated and the results of these studies showed a time and concentration dependent increase in haemolysis.[150]

Although the effects of Pb and Cd are well described, information on the effects of Cr and Al on the erythrocyte membrane is limited. In the present study Cr and Al were found to cause haemolysis although to a lesser degree than Pb and Cd.

4.2 THE EFFECTS OF AAPH AND METALS ON ERYTHROCYTE MORPHOLOGY

4.2.1 EFFECT OF AAPH – INDUCTION OF OXIDATIVE DAMAGE

In order to identify and describe the sequential morphological changes that erythrocytes undergo following exposure to oxidants, human erythrocytes were exposed to 300mM of AAPH for 16hrs at 37ºC. At times, 1, 2, 4, 6, 8 and 16 hrs, the degree of haemolysis was measured (Figure 4.2) and changes to erythrocyte morphology was determined with SEM (Figure 4.4). Erythrocyte morphology was described and classified according to the classification in Figure 4.3.

AAPH is an azo compound which generates free radicals by thermal decomposition in an aqueous state and was used as a source of peroxyl radicals.[151] During AAPH decomposition, molecular nitrogen and free radicals are formed. The radicals then react with oxygen to produce peroxyl radicals which attack the erythrocyte membrane.[152] AAPH has a half-life of approximately 175 hrs at 37°C and neutral pH, thus the rate of free radical production is basically constant within the first few hrs in solution.[153]

Oxidative damage causes nucleated cells to undergo cell death via apoptosis. Mature erythrocytes do not contain nuclei or organelles but also have the ability to undergo self-destruction similar to nucleated cells. Although the process of apoptosis is well described for nucleated cells, less is known about this process in anucleate cells. In nucleate cells ROS activates the mitochondria mediated intrinsic pathway of apoptosis. Although mature erythrocytes do not contain mitochondria and nuclei, these cells do undergo several changes associated with apoptosis such as cell shrinkage, membrane blebbing, and shape transformation from a discocyte to a spherocyte which is associated with the externalization of phosphatidylserine.[154,155] Gyawali[134] *et al*. (2015) observed that an increase in oxidative stress and inflammation caused morphological changes to human erythrocytes.[134] When exposed to oxidants and inflammatory agents erythrocytes lose their discoid shape and progress to become echinocytes and finally spherocytes/ sphero-echinocytes and can be classified according to Figure 4.3. These morphological changes are due to changes that occur to the structure of the cytoskeleton as a result of oxidative stress.[156-158]

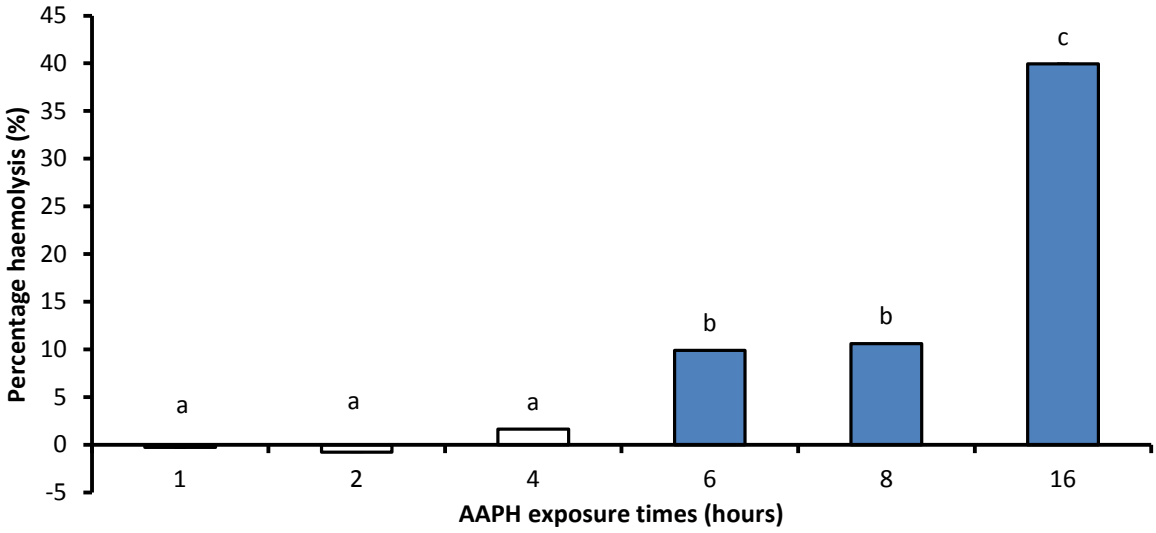


Figure 4.2: A time based study on the ability of 86mM AAPH on erythrocyte haemolysis. Data is an average of three experiments with three data points each. Standard error of the mean < 0.02%. Means with different letters are significantly different, p≤0.001. Solid blue bars indicates signiicant levels of AAPH induced haemolysis.

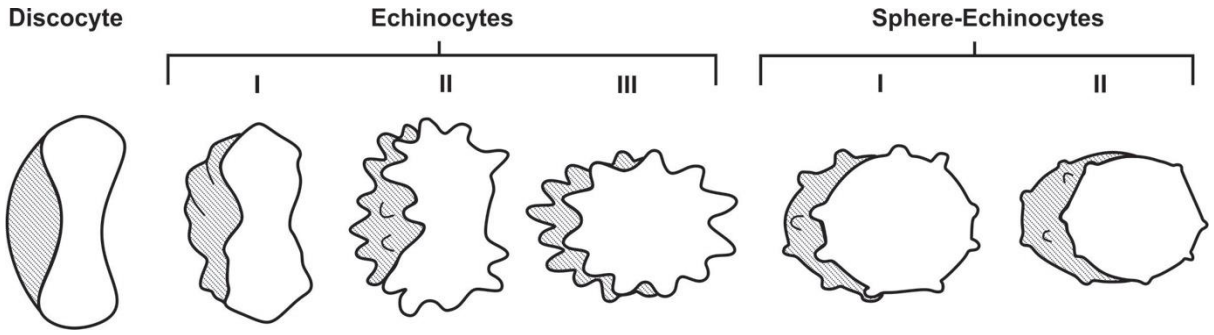


Figure 4.3: Morphological changes associated with eryptosis. Adapted from http://vetbook.org/wiki/cat/images/4/4f/EchinoFig1.jpg. [159]

At time intervals of 1, 2 and 4 hrs the measured levels of haemolysis was < 5%. At 6 and 8 hrs, the percentage of haemolysis was 10% and after 16 hrs 40% haemolysis occurred (Figure 4.2). Time dependent morphological changes due to increased oxidative damage was observed (Figures 4.4) from images A through to H. At 0 hrs, the erythrocytes had a typical biconcave discoid shape associated with discocytes. At 1, 2 and 4 hrs, AAPH induced the formation of echinocytes which have characteristic rounded projections or spicules. At 1 hr exposure type I or early echinocyte formation occurs while at 2 and 4 hrs primary echincyte formation (type II and III respectively) was observed. These structures were associated with haemolysis of only ± 5%.

As echinocytosis progresses the spicules become more numerous and eventually bud off in the form of extracellular vesicles which consist of plasma membrane material (Figure 4.4 D),

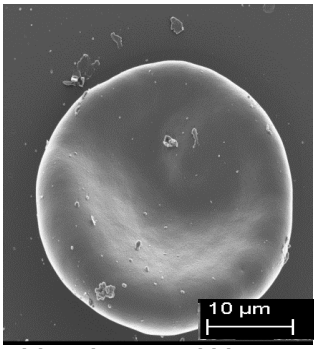
and at 4 hrs this is associated with ± 10% haemolysis. At the same time the spicules also begin to swell and become more and more spherical (Figures 4.4 E & F). The spherical erythrocytes with spicules are known as sphere-echinocytes (Figure 4.4 G) and types I and II have been described. Once the vesicles have budded off spherical smooth erythrocytes form (Figure 4.4 H). This type of erythrocyte has a smaller volume and surface area than the original discocyte and echinocyte forms. These spherocytes (Lim 2002) or a sphere-echinocyte type II (Figure 4.3) is associated with 40% haemolysis after 16hrs.

Erythrocytes undergoing eryptosis have a plasma membrane that is essentially intact and only with further exposure to ROS a more necrotic phenotype develops with increased permeability and consequently leakage of Hb, and this occurs at the stages of spheroechinocyte and spherocyte formation.

Erythrocyte cell membranes are usually firm and have little channel activity. In cases of oxidative stress, channel activity increases and the tightness of the membrane is compromised. Osmotic and oxidative stress causes calcium (Ca^{2+}) permeable cation channels in erythrocytes to open which increases cytosolic Ca^{2+} levels that are responsible for the breakdown and externalization of phosphatidylserine associated with eryptosis.[160-163] Oxidative stress was also observed to cause cell shrinkage which is most likely due to the activation of the Ca^{2+} sensitive K^+ channels present in the erythrocyte membrane. This leads to hyperpolarization of the erythrocyte membrane and thus the consequent loss of K^+ ions.[160,161] In addition to eryptosis, the activity of not only the Ca^{2+} channels but also that of the K^+ channels, anion channels, taurine channels as well as the Na^+/H^+ exchange channels are affected. Oxidative stress triggers the opening of the above non-selective cation channels which in turn has an effect on the permeability of the cell membrane as well as membrane integrity.[160-163]

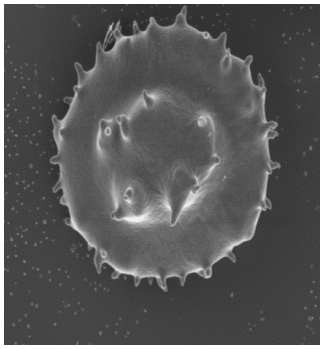
Similar to the peroxyl radicals generated by AAPH, $OH^{\bullet}$ radicals formed from H_2O_2 via the Fenton reaction catalysed by Fe^{2+} would also have an adverse effect on erythrocyte morphology and membrane permeability. H_2O_2 has been reported to induce echinocytosis.[164] Oxidants such as acrolein and phenylhydrazine were also found to cause echinocytosis.[165] Longer incubation times caused increased haemolysis and severe damage to the erythrocyte membranes, including pitting and the formation of holes.

A



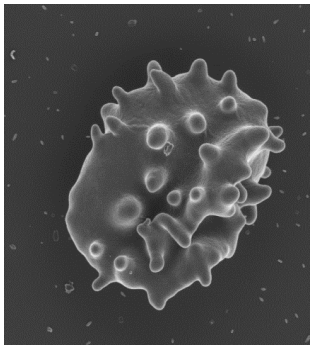
Control (PBS): Normal biconcave morphology. Discocyte

B



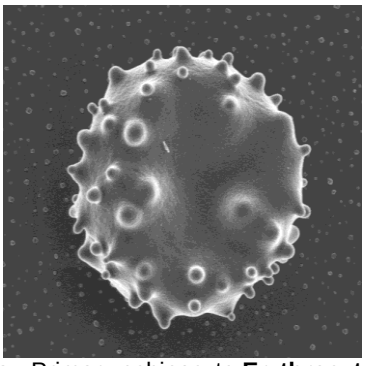
1 hr: Early echinocyte formation: Spicules form on erythrocte membrane. **Echinocyte, type I, ± 5%H**

C



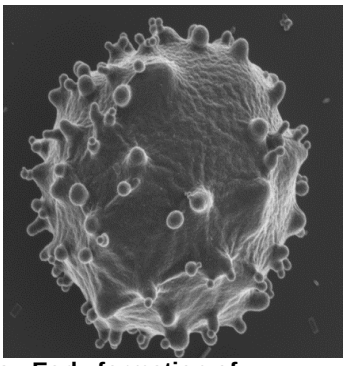
2 hrs: Primary echinocyte: Erythrocyte, deformation. Spicules beome nodule-like projections. **Echinocyte, type II, ± 5%H**

D



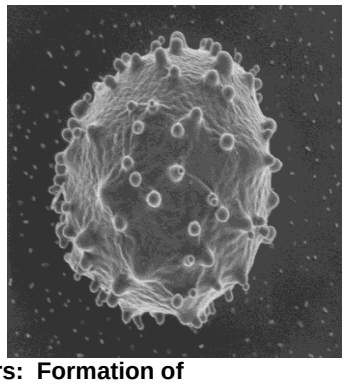
4 hrs: Primary echinocyte **Erythrocyte start to swell.** Echinocyte, type III, ± 5%H

E



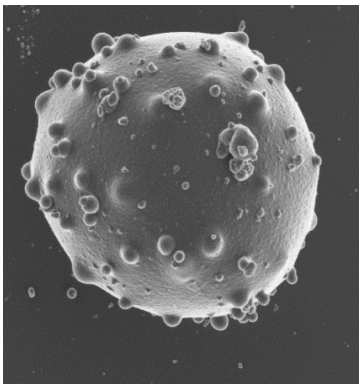
6 hrs: Early formation of spheroechinocytes: Erythrocyte continue to swell and membrane starts to detatch. **Spheroechinocytes , type I, ± 10%H**

F



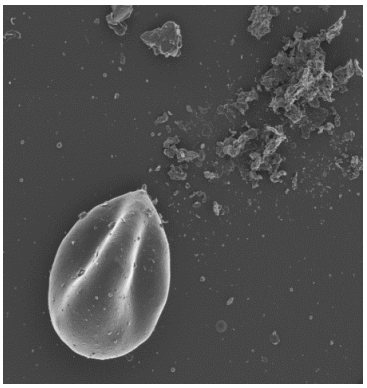
8 hrs: Formation of spheroechinocytes: Erythrocyte continue to swell and membrane detaches further. **Spheroechinocytes, type I, ± 10%H**

G



16 hrs: Spheroechinocyte **Some erythrocytes become spherical, with with a few nodule like projections still present.** Spheroechinocytes , type II, ± 40%H

H



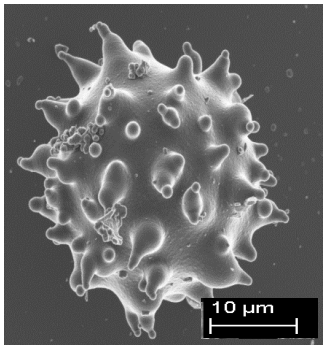
16 hrs: Spherocyte: Some erythrocytes burst open, loss of content and cell death of the cell**.** **→ → → progression, > 40%H**

<u>Figure 4.4:</u> Effects of oxidant AAPH on morphology of human erythrocytes. SEM images of (A) Control treated with PBS, the rest of the erythrocytes were treated with 86mM AAPH for (B) 1, (C) 2, (D) 4, (E) 6, (F) 8 and (G), (H) 16 hrs. All images are the same magnification, bar in A = 10µm.

4.3 EFFECTS OF METALS ON ERYTHROCYTE MORPHOLOGY

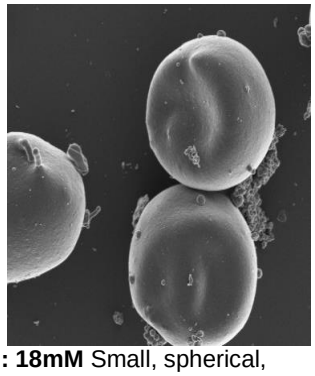
Once the sequential changes in erythrocyte morphology was identified, erythrocytes were then exposed to Pb, Cd, Cr and Al at 13mM, 18mM, 30mM and 63mM respectively for 16 hrs. All metal concentrations induce H50%. It is predicted that if $OH^{\bullet}$ radical formation was the only mechanism of action, the observed erythrocyte morphology would be similar to that presented in Figure 4.4 G and H. Following exposure to Pb, (Figure 4.5 A), the erythrocytes had the typical morpology of echinocytes, type II. For Cd, completely smooth spherocytes form and this is structurally similar to the spherocytes observed in Figure 4.4 H. Although in Figure 4.4 H the spherocyte has burst. Following exposure to Cr, the typical morphology is that associated with echinocytes, type III. Al also causes the formation of echinocytes, type III although the observed changes are more advanced than that observed for Cr i.e fewer and smaller spicules. The morphology observed for Pb, Cr and Al are not typical of that for H50% (Figure 4.2 and 4.4 G and H) which may imply that mechanisms other than membrane effects are involved that leads to haemolysis. Only Cd was found to induce a typical morphology assicated with oxidative damage that leads to H50%. Pb, Cr and Al at: 13mM; 30mM and 63mM concentrations respectively produce echinocytes associated with low levels of haemolysis and that Cd at 18mM produces spherocytes associated with high levels of haemolysis. Differences following exposure of erythrocytes to Pb, Al and Cr may be that these metals have additional cellular targets such as inhibition of antioxidant enzymes and the depletion of GSH.

A



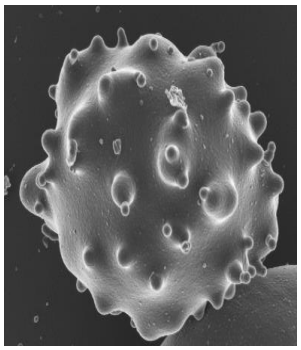
Pb: 13mM Deformation and swelling of erythrocyte, spicules form on the surface of the erythrocyte. **Echinocyte, type III**

B



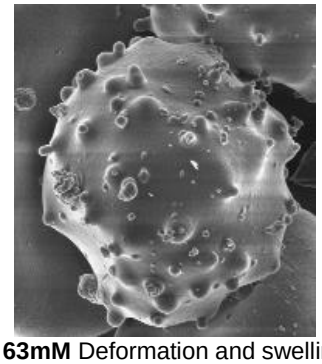
Cd: 18mM Small, spherical, erythrocytes with no spicules on the surface of the cells. **Spherocytes**

C



Cr: 30mM Deformation and swelling of erythrocyte, spicules form on the surface of the erythrocyte. **Echinocyte, type III**

D



Al: 63mM Deformation and swelling of erythrocyte, spicules form on the surface of the erythrocyte. **Echinocyte type I**

Figure 4.5: Predominant morphological effect of 13, 18, 30 and 63 mM of Pb, Cd, Cr and Al respectively on erythrocyte morphology after 16 hrs of incubation at 37°C. All images are the same magnification, bar in A = 10µm.

4.4 ABILITY OF PB, CD, CR AND AL TO CATALYSE THE FENTON REACTION

The ability of each metal to act as catalysts in the Fenton reaction was then determined using a modification of the HORAC assay. The principle of the HORAC assay is based on the oxidation of fluorescein by $OH^{\bullet}$ radicals generated from metal catalysis of H_2O_2. In this assay the free radicals generated by H_2O_2 quench the fluorescence of the fluorescein over time.[28,137] Usually this assay is used to evaluate the antioxidant activity of compounds or plant extracts, which scavenge radicals thereby preventing the quenching of fluorescein fluorescence. Usually the reaction is catalysed by Co, instead Co was replaced by Pb, Cd, Cr or Al at a 1/6 of the concentration used to induce H50%.

The results of this assay showed that all the metals; Pb, Cd, Cr, Al and Co (which was used as a control), catalysed, to varying degrees, the conversion of H_2O_2 with the formation of $OH^{\bullet}$ radicals (Figure 4.6). The fluorescence units quenched per mM metal was then calculated and was the highest for Cr > Cd > Pb > Al. Both Pb and especially Al were poor catalysts of the Fenton reaction. Therefore observed echinocyte formation was partially due to $OH^{\bullet}$ formation generated by the Fenton reaction.

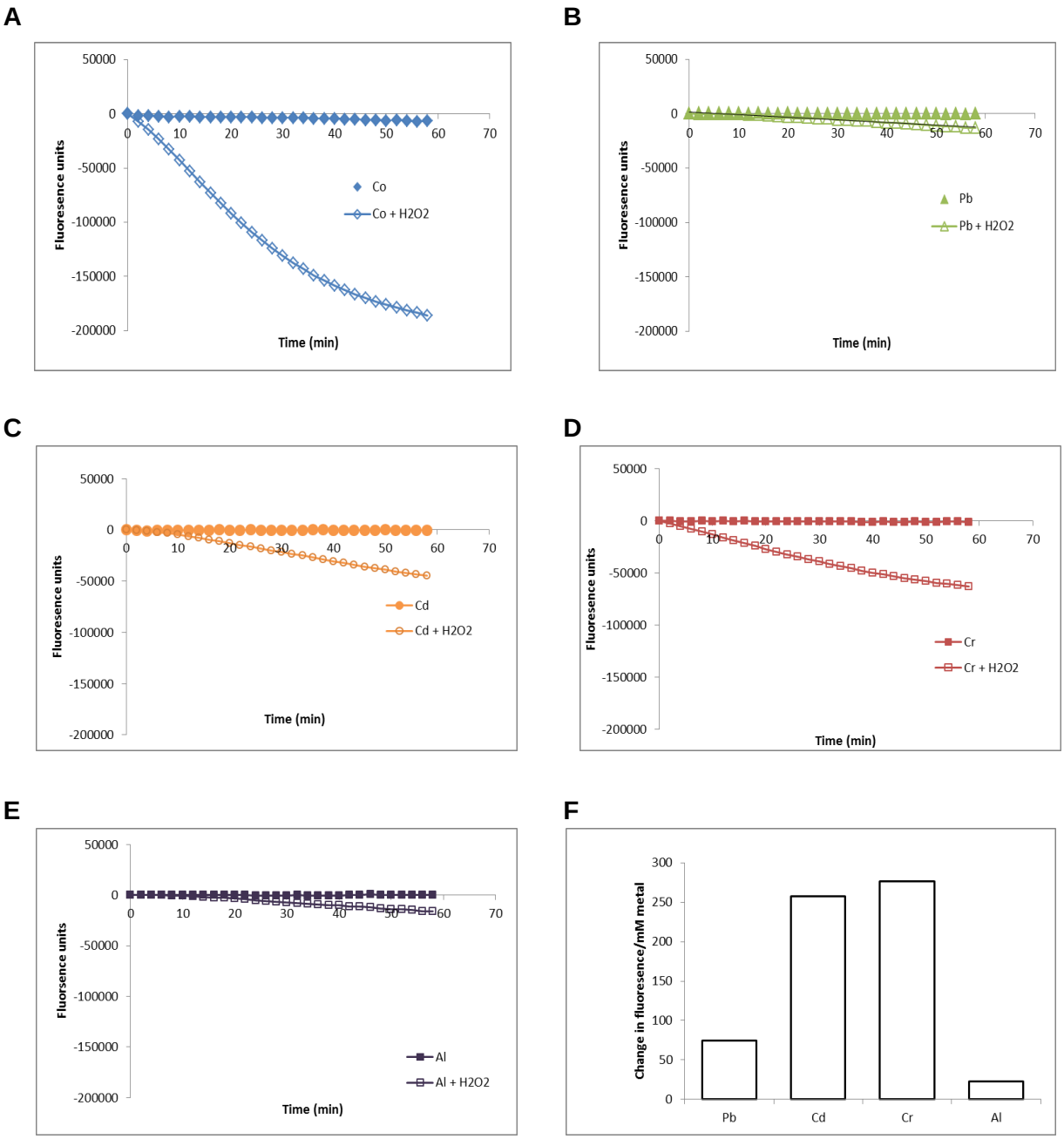


Figure 4.6: Changes in fluorescence units seen by Co 0.46mM, Pb 2.25mM, Cd 3mM, Cr 4.75mM and Al 11mM shown in A-E. F indicates the changes in fluorescence seen between the different metals. Data is an average of two experiments with three data points each. Standard error of the mean < 0.02%.

4.5 OXIDATIVE DAMAGE

H_2O_2 which is produced by various reactions and in several cellular compartments acts as a secondary messenger molecule and regulates several important cellular processes. Excessive amounts of H_2O_2 can be toxic to cells and when in the presence of metals with low oxidative states, it can be reduced to form $OH^{\bullet}$ radicals.[166] In a cellular environment H_2O_2 levels are tightly controlled by antioxidant enzymes such as catalase, thereby protecting cells such as erythrocytes against oxidative damage. Although metals such as Cd and Cr were found to effectively act as catalysts in the Fenton reaction, it is only in a cellular environment that the actual cellular effect can be determined.

4.5.1 ABILITY OF METALS TO INDUCE OXIDATIVE DAMAGE IN ERYTHROCYTES

The DCFH-DA assay is an assay widely used to detect oxidative damage caused by ROS and RNS.[140] This method was used in this study to determine if ROS contributes to erythrocyte haemolysis. AAPH was used as a control inducing 100% oxidative damage. Metal concentrations that induce H50% were evaluated. For the haemolysis assay, erythrocytes were exposed to each metal for 16 hrs and to DCFH-DA for 1hr, where after a time dependent increase in fluorescence was measured. The rate of increase in fluorescence was measured and was expressed as percentage oxidative damage compared to the control, AAPH that induced 100% damage.

Pb caused 61.74% oxidative damage while no increase in ROS was observed for Cd, Cr and Al (Figure 4.7). Evaluation of a typical example of a DCFH-DA curve (Figure 4.8) reveals no ROS formation for Cd, Al and Cr over the time period evaluated. The measured fluorescence for Al and Cr over the period evaluated (2-18 min) was less than the blank. The DCFH-DA is dependent on intracellular esterase enzymatic activity and these results indicate that both metals in varying degrees may inhibit enzyme activity.

In a study conducted by Xu[167] *et al.* (2008), ROS produced by Pb acetate in mice liver cells was measured using the DCFH-DA assay as an indicator of oxidative stress.[167] Mice were exposed to Pb acetate orally for 4 weeks at doses of 0, 10, 50 and 100mg/kg of body weight every 48 hrs. The results showed that Pb acetate statistically increased the levels ROS in mice.[167] In the present study, Pb caused significant amounts of ROS induced damage at the highest concentration tested (13mM).

In contrast to the present study, Jing[168] *et al.* (2012) reported that 5µM Cd increased ROS production evaluated with the DCFH-DA assay in bronchial epithelial cells.[168] In the present

study, although Cd was shown to catalyse ROS formation, the DCFH-DA assay shows a lack of ROS formation. For metals to catalyse the Fenton reaction there must be sufficient levels of H_2O_2. High levels of the enzyme catalase found in erythrocytes converts H_2O_2 to H_2O and O_2 and this may account for the lack of ROS that forms following exposure of erythrocytes to Cd. Although a short-term increase in ROS production was not observed in the present study, SEM evaluation of the morphology of erythrocytes following 16 hr exposure to Cd indicates that ROS associated morphological changes do occur.

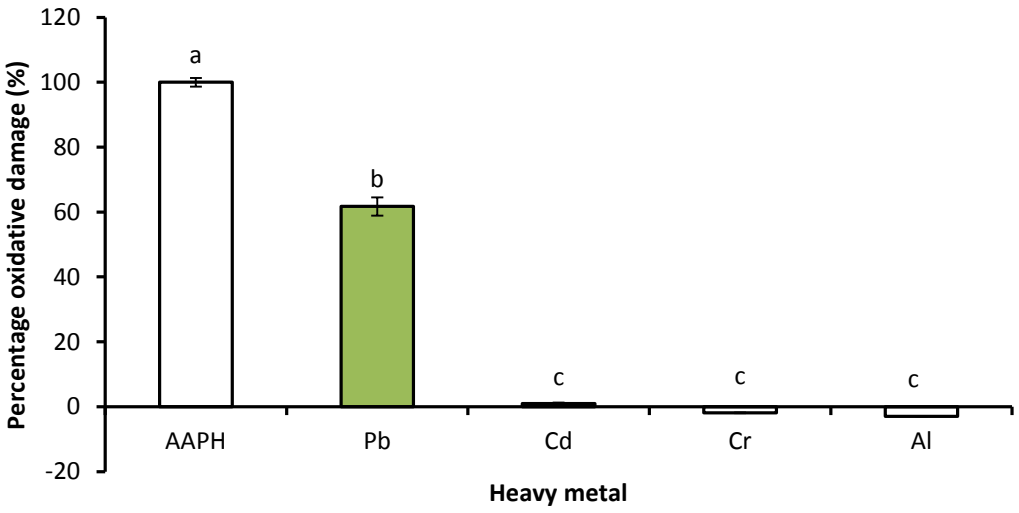


Figure 4.7: Percentage oxidative damage induced by 13mM Pb,18 mM Cd, 30mM Cr and 63mM Al measured with the DCFH DA assay. AAPH was used as the control, 100% oxidative damage. Data is an average of three experiments. Standard error of the mean < 0.02%. Means with different letters are significantly different, p≤0.001. Green indicates the metals that cause oxidative damage.

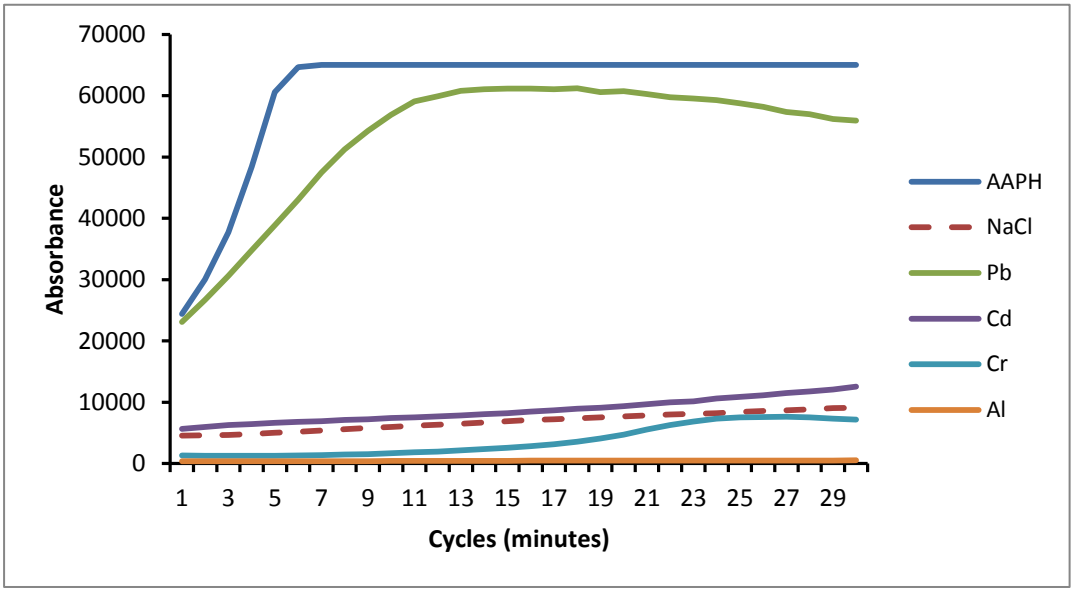


Figure 4.8: Example of a typical curve generated with the DCFH-DA assay. Erythrocytes were exposed to 20mM AAPH, 115mM NaCl, 13mM Pb, 18mM Cd, 30mM Cr and 63mM Al and the change in fluorescence was measured for 1 hr. Each cycle represents 2 min. Data is an average of three experiments with three data points each. Standard error of the mean < 0.02%.

Using the DCFH-DA assay, Ye[169] *et al.* (1999) found that Cr(VI) was able to generate ROS in human epithelial cells.[169] Analysis of the distribution of the dye with fluorescence microscopy revealed that there was an increase in both $OH^{\bullet}$ and H_2O_2 in the mitochondria of treated cells.[169] It was concluded that ROS formation in these cells was dependent on mitochondrial pathways and as erythrocytes lack mitochondria this may account for the lack of ROS mediated toxicity, observed in the present study.

Vota[170] *et al.* (2012) observed an increase in ROS generated in erythrocytes exposed to Al for 21 days.[170] Using erythrocytes in the present study it was found that there was no short-term ROS formation. Lack of an oxidative effect observed for Al and Cr with SEM or the DCHF-DA assay may also be a function of the concentration used and exposure time.

4.6 HEAVY METAL INTERACTIONS WITH GSH

Besides radical formation, metals may cause toxicity by depleting cellular antioxidant elements such as GSH. Depletion of GSH reduces a cell and tissues ability to scavenge radicals. GSH is a tripeptide consisting of glutamate, cysteine and glycine and the functional – (sulfhydryl) SH group of cysteine plays a major role in heavy metal binding. Studies have shown that rats exposed to Pb have decreased GSH in organs such as the brain and liver.[18,60,171]

4.6.1 DIRECT METAL – GSH BINDING

GSH levels can be quantified using DTNB or Ellman's reagent and the formation of a yellow product can be quantified. This assay can be used to quantify GSH levels in whole blood, serum, lung samples, cerebrospinal fluid as well as tissue and cell extracts.[80,172] Binding of a metal to GSH would result in a decrease in absorbance. In addition to 1mM GSH an excess of Pb, Cd, Cr and Al (13mM Pb, 18mM Cd, 30mM Cr and 63mM Al) was added and mixed well. The amount of free, unreacted GSH was then quantified with Ellman's reagent. At the concentrations evaluated, Cd, Cr and Al bound a 100% GSH while the binding ability of Pb was only 16% compared to >94% for Cd, Cr and Al (Figure 4.9). Therefore, it appears that Pb induces oxidative damage possibly via ROS formation while Cd, Cr and Al preferably bind GSH. As all metals were added at concentrations greater than GSH, it would be of value to determine the binding effect of increasing concentrations of metals in order to determine the specific binding capacity of Cd, Cr and Al.

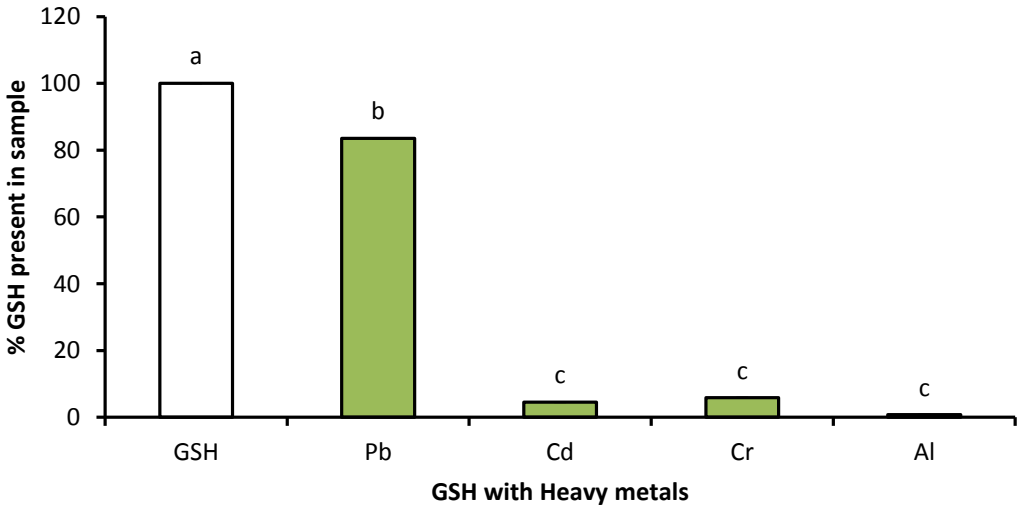


Figure 4.9: Ability of 13mM Pb, 18mM Cd, 30mM Cr and 63mM Al to bind 1mM GSH. Data is an average of three experiments with three data points each. Standard error of the mean < 0.02%. Means with different letters are significantly different, p≤0.001**.**

4.6.2 EFFECTS OF METALS ON ERYTHROCYTE GSH LEVELS

The GSH pathway consists of GSH, GSSG and two key enzymes, GR and GPx that neutralizes H_2O_2 by removing both hydrogen atoms from two GSH molecules which results in the formation of two H_2O molecules and one molecule of GSSG. GR then regenerates GSH from GSSG as shown in Figure 2.2.[96] Binding of metals to GSH will result in a decrease in intracellular GSH levels. Inhibition of GR will result in the accumulation of GSSG while inhibition of GPx causes the accumulation of GSH.

Erythrocytes were exposed for 16 hrs to 13mM, 18mM, 30mM and 63mM of Pb, Cd, Cr and Al respectively. No significant decrease in GSH levels was measured (Figure 4.10). A dosage dependant increase in erythrocyte GSH levels was only measured for Pb at 7-13mM as well as 27 and 30mM Cr. This implies that Pb inhibits GPx while Cr inhibits GPx but also binds GSH.

The effect of heavy metals, Ag(I), Hg(II), Cu(II) and Pb(I) on GSH and associated enzymes in an erythrocyte model was found to cause a decrease in GSH content and in GPx activity. The researchers could not determine the effect of Cu(II) on GPx as Cu(II) rapidly oxidises GSH and prevents accurate estimation of enzyme, although previous studies have shown that Cu(II) inhibits GPx.[173]

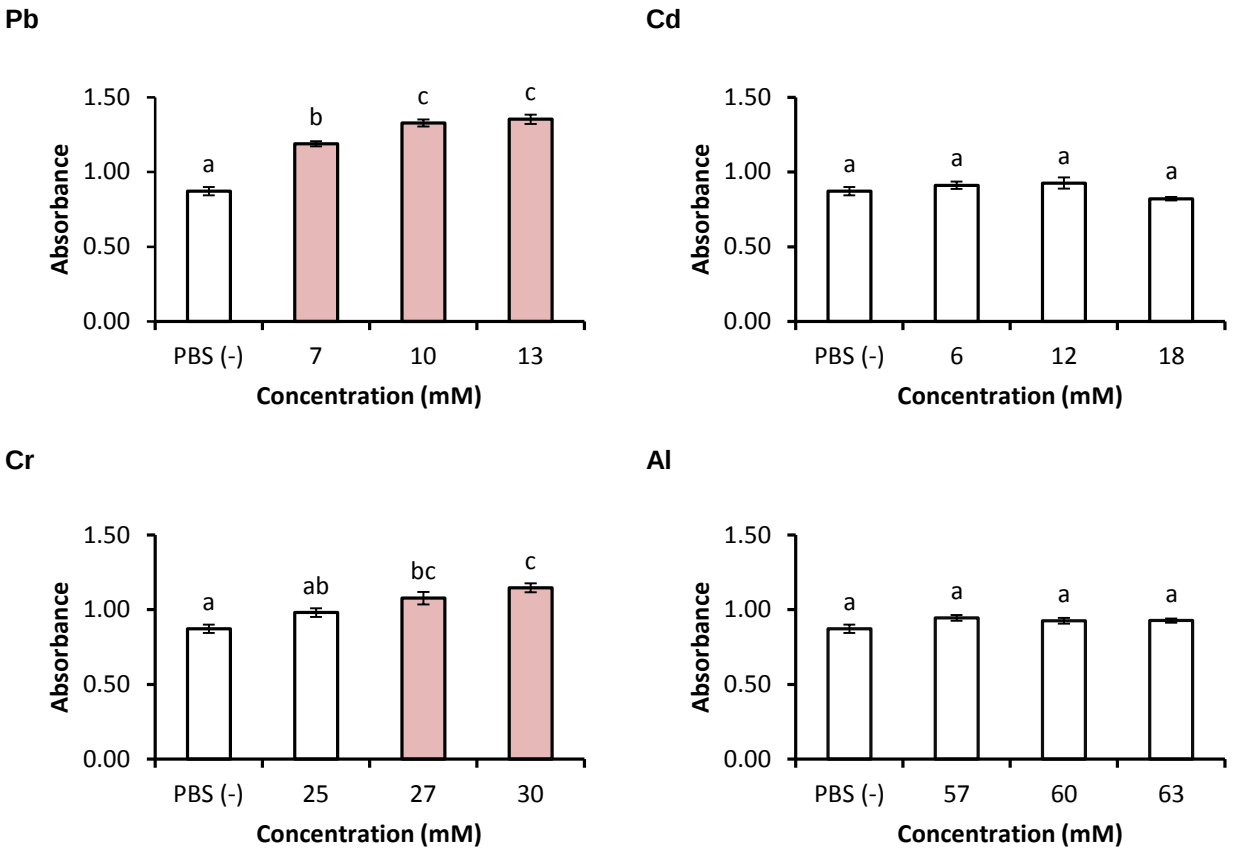


Figure 4.10: GSH levels in erythrocytes exposed to Pb (7-13mM), Cd (6-18mM), Cr (25-30mM) and Al (57-63mM). Data is an average of three experiments with three data points each. Standard error of the mean < 0.02%. Means with different letters are significantly different, p≤0.001.

A known effect of GSH is that it protects cells from oxidative stress. Any changes in GSH levels (an increase or decrease) indicates that the oxidative status has been disturbed. When cells are oxidatively challenged GSH synthesis may increase. With continued stress, the GSH pathway cannot efficiently supply the demand, and consequently GSH depletion occurs. Cd toxicity has been reported to cause an initial increase in GSH levels followed by depletion of GSH. A similar effect was also observed for Hg and this was also associated with an increase in H_2O_2 levels.[18] In the present study Pb(II) was found not to bind GSH but did cause an increase in GSH levels indicating that Pb(II) is likely to have the similar mechanism to Hg where GSH levels are initially raised together with increased ROS formation. Cr(III) showed GSH binding abilities and like Pb(II) showed an increase in GSH levels. Cd and Al had no significant effects on GSH levels.

To summarize, Table 4.2 shows metal concentrations that induce H50%, the associated change in erythrocyte morphology and GSH levels. The possible mechanism related to the ability to catalyse the Fenton reaction and to bind GSH is also indicated.

Pb causes echinocyte formation, and is a poor catalyst of the Fenton reaction, although it does induce ROS formation. Pb does not bind GSH but does cause the accumulation of GSH possibly due to GPx inhibition. Both Cd and Cr appear to have similar effects as both are effective catalysts of the Fenton reaction, do not cause ROS formation but do bind GSH. However, Cd and Cr have different morphological effects on the cell membrane. Al only causes haemolysis at high concentrations, and only induced echinocyte, type I formation. Al is a poor catalyst of the Fenton reaction and does not induce ROS formation in erythrocytes. Al does bind GSH but has no effect on intracellular levels in erythrocytes.

This preliminary study identified erythrocyte targets but also provided an indication that these metals have multiple targets and effects are a function of concentration and exposure time.

Table 4.2: Summary of the effects of heavy metals, Pb, Cd, Cr and Al

EFFECT	Pb	Cd	Cr	Al
H50% (mM)	16.00	33.38	47.83	81.26
Erythrocyte morphology	Echinocyte, type III	Spherocytes	Echinocyte, type III	Echinocyte, type I
Oxidative damage				
Radical formation				
Change in fluorescence / mM	74.80	252.17	276.12	22.52
Erythrocyte oxidative damage	Time dependent	NC	NC	NC
GSH effects				
Percentage GSH binding	16.48	95.53	94.14	99.18
Erythrocyte GSH levels	Increased	NC	Increased	NC

NC = No change

B: PROTECTIVE EFFECTS OF POLYPHENOLIC, FLAVONOIDS AND VITAMIN DERIVED ANTIOXIDANTS ON METAL INDUCED OXIDATIVE DAMAGE

Heavy metal binding of antioxidants such as polyphenols and depletion of dietary antioxidants results in cells and tissue being more susceptible to oxidative damage. Nevertheless, this is also a mechanism whereby cells and tissue can be protected against oxidative damage. This is often the first line of defence where dietary polyphenols scavenge heavy metals prior to them reaching cellular and tissue targets. The mechanisms involved will be discussed in greater detail in sections 4.7 to 4.12.

In this study several dietary antioxidants that occur in foods and that are bio-available were identified. Two of these were gallic acid and catechin. Gallic acid is widely found in black and green tea, red wine, nuts, plants like sumac and witch-hazel and fruits for example grapefruit, grape seed oil, dates, apples and pomegranates.[174] Gallic acid and isoflavones are the best absorbed polyphenols followed by catechins, flavanoids and quercetin glucosides.[175] Catechin is a major antioxidant found in black tea, apples, pears, grapes, chocolate and red wine.[176] The absorption of catechins differs, with pure catechins being absorbed better in humans, while catechins that have undergone galloylation are absorbed less effectively. In order to evaluate how antioxidants reduce metal mediated $OH^{\bullet}$ formation the effects of caffeic acid and quercetin were also determined. Caffeic acid is primarily found in high concentrations in coffee but is also found in a number of food sources including bean sprouts, chia seeds, blueberries,

plums, kiwis, cherries, apples, artichokes and wine.[178] Quercetin is found in a variety of food sources including teas, apples, capers, berries, kale and onions.[179]

Identified antioxidant vitamins were Vitamin E and C. Vitamin E and its derivatives are found in several food sources such as walnuts, soy, corn, grape seed, almond, sunflower and palm oil. Vitamin E and its derivatives inhibit lipid peroxidation by scavenging lipid peroxyl radicals, quicker than these radicals can react with fatty acid side-chains or membrane proteins.[177] Vitamin E is water insoluble and therefore Trolox, an α-tocopherol derivative was used in this study. The hydrophobic phytyl group of α-tocopherol is replaced with a hydrophilic carboxylic group to produce Trolox, which is water soluble. Trolox can terminate two peroxidation chains (chain breaking antioxidant), and can also quench and react with singlet O_2.

Ascorbic acid is found in a variety of fruits and vegetables including strawberries, oranges, lemons, mandarins, kiwi fruit, grapefruit, mangoes, cauliflower, broccoli, cabbage, garlic and tomatoes.[180] Ascorbic acid is an essential vitamin but is also a strong reducing agent and scavenger of oxidising free radicals and harmful oxygen-derived species such as $OH^{\bullet}$, H_2O_2, and $^1O_2$. As a strong reducing agent it is willingly reversibly oxidised to dehydroascorbic acid. As a vitamin, ascorbic acid is essential for the synthesis of neurotransmitters and hormones, it participates in neurochemical, chemical and enzymatic reactions, enhances Fe absorption, cell growth and differentiation, is essential for wound healing, and maintains blood vessel health and integrity.[98]

An aim of this part of this study was to determine if the identified antioxidants have a protective effect against heavy metal induced toxicity. Antioxidants were chosen based on abundance in the diet (catechin, gallic acid and Trolox) and type (flavonoid, polyphenol and vitamins). Antioxidants can protect the cells from damage and are able to decrease the toxic effect of these metals by using various mechanisms. These mechanisms were evaluated and the results were presented in sections 4.7 to 4.12.

4.7 ABILITY OF ANTIOXIDANTS TO PREVENT METAL INDUCED ERYTHROCYTE HAEMOLYSIS

An antioxidant is defined by Halliwell and Gutteridge[177] as “any substance that when present at low concentrations compared with that of an oxidizable substrate, significantly delays or inhibits oxidation of that substrate”.[177] Erythrocytes were exposed to 0.2mM of gallic acid, Trolox and catechin and then increasing concentrations of each metal. The gradient of each line was calculated and compared to that of erythrocytes only exposed to metals (Table 4.1).

Pre-incubation of the erythrocytes with antioxidants caused a significant decrease in measured percentage haemolysis. The line equations for each combination and the fold decrease in haemolysis is presented in Table 4.3.

Table 4.3: The effect of antioxidants on the percentage haemolysis induced by Pb, Cd, Cr and Al

Metal + antioxidant	Equation		Fold decrease*
Pb + gallic acid	y = 2.01x + 0.57	R^2 = 0.992	1.5
Pb + Trolox	y = 1.73x - 1.25	R^2 = 0.959	1.8
Pb + catechin	y = 3.59x + 1.03	R^2 = 0.993	1.2
Cd + gallic acid	y = 1.67x - 10.35	R^2 = 0.985	1.4
Cd + Trolox	y = 1.40x - 7.52	R^2 = 0.990	1.3
Cd + catechin	y = 1.91x - 9.35	R^2 = 0.998	0.9
Cr + gallic acid	y = 4.77x - 109.25	R^2 = 0.994	**1.9**[e]
Cr + Trolox	y = 5.03x - 117.93	R^2 = 0.989	1.7
Cr + catechin	y = 3.09x - 77.55	R^2 = 0.772	**2.5**[c]
Al + gallic acid	y = 2.67x - 123.04	R^2 = 0.774	**3.2**[b]
Al + Trolox	y = 3.66x - 192.43	R^2 = 0.953	**2.0**[d]
Al + catechin	y = 0.73x - 36.577	R^2 = 0.974	**10.5**[a]

Compared to gradients Table 4.1. Letter a-e indicate the most effective metal – antioxidant combinations.

Gallic acid, Trolox and catechin decreased the degree of haemolysis induced by all metals by variable degrees. Compared to the divalent metals, antioxidants bound more effectively to the trivalent metals with a 1.7-2.5 fold and 2.0 – 10.5 fold decrease in haemolysis for Cr and Al respectively. Of all combinations, catechin was the most effective in inhibiting the haemolytic effects of Cr, resulting in a 10.5 fold decrease in the measured levels of haemolysis. This implies that catechin found in tea can effectively reduce the toxicity of Al. This is of specific value as Al toxicity is associated with the development of Alzheimer's disease.

Tagliafierro[181] *et al.* (2015) investigated the protective effects of the phenolic acid, hydroxytyrosol (HT) found in olive oil against Hg induced haemolysis. Hg concentrations of 40μM and 80μM induced a dosage and time dependent increase in haemolysis. HT at a concentration of 10μM significantly inhibited haemolysis.[181] Likewise, Oficioso[182] *et al.* (2016) also observed that HT protected human erythrocytes against Hg induced haemolysis and eryptosis.[182] In the present study, low concentrations of the phenolic acid, gallic acid effectively inhibited Pb, Cd, Cr and Al induced haemolysis.

Catechin is a flavonoid found in tea. Liu and Huang[183] (2015) evaluated the antioxidant effects of black tea extracts by using the erythrocyte haemolysis assay, plasma oxidation and cellular

antioxidant activity (CAA) assay. Black tea was found to be an effective antioxidant against oxidative stress and decreased ROS induced haemolysis.[183] In the present study catechin reduced the levels of haemolysis induced by each metal. The greatest effect was observed for Al (57 – 63mM), where 0.2mM catechin induced a 10.5-fold decrease in haemolysis.

4.8 PROTECTION AGAINST RADICAL FORMATION

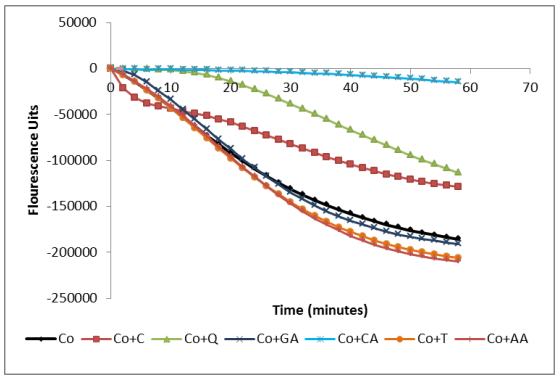
4.8.1 ABILITY OF ANTIOXIDANTS TO INHIBIT THE FENTON REACTION

The Fenton reaction requires metals to serve as catalysts to convert H_2O_2 to $OH^{\bullet}$. Antioxidants such as phenolic acids, flavonoids and antioxidant vitamins can either directly bind the metals thereby inhibiting the Fenton reaction or the antioxidants do not bind the metals but directly scavenge the formed $OH^{\bullet}$. The ability of the antioxidants to reduce $OH^{\bullet}$ mediated quenching of fluorescein fluorescence was evaluated. In this part of the study the effect of quercetin, caffeic acid and ascorbic acid was also investigated. As a result the effect of two phenolic acids, flavonoids and vitamins on the ability of Co (control), Pb, Cd, Cr and Al to generate $OH^{\bullet}$ radicals was determined and this is presented in Figure 4.11. Three effects could be identified and these were a pro-oxidant effect associated with an increase in radical formation, no effect or an antioxidant effect associated with a decrease in radical formation.

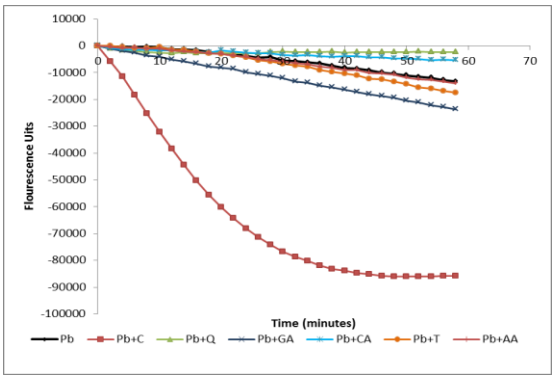
The observed effects, between metals, Pb, Cd, Cr and Al and the antioxidants evaluated is presented in Figure 4.11 while in Figure 4.12 the effectivity of each antioxidant is summarised in Table 4.4.

Although Pb is a poor catalyst of the Fenton reaction, when combined with catechin a strong pro-oxidant effect is observed. Also in combination with Cd, catechin also has a strong pro-oxidant effect. Lessor effects were observed with Cd and Cr in combination with catechin and caffeic acid as well as gallic acid in combination with Al. A pro-oxidant effect has been reported for several polyphenols and these include catechin, gallic acid, caffeic acid, quercetin, ferulic acid and activity is a function of concentration and experimental conditions.[184]

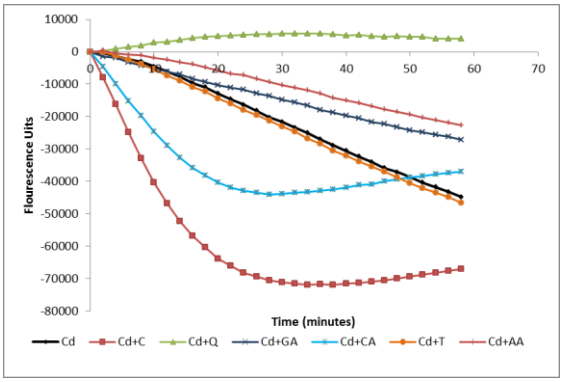
Co



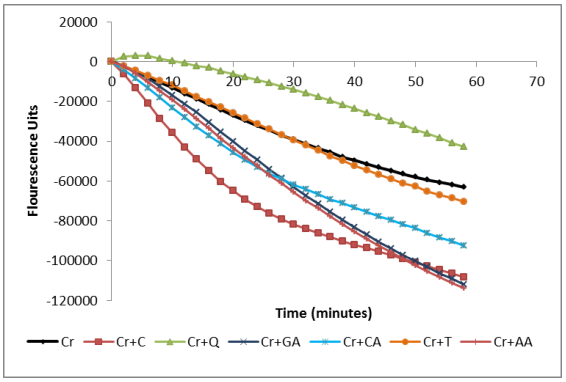
Pb



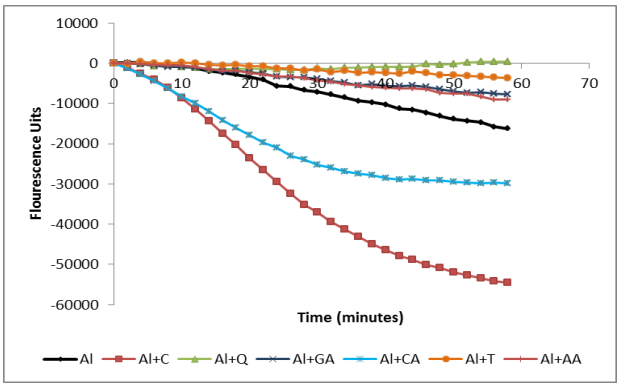
Cd



Cr

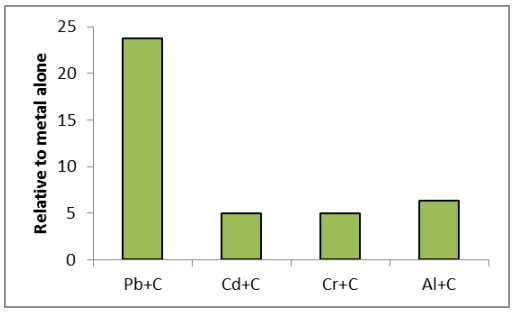


Al

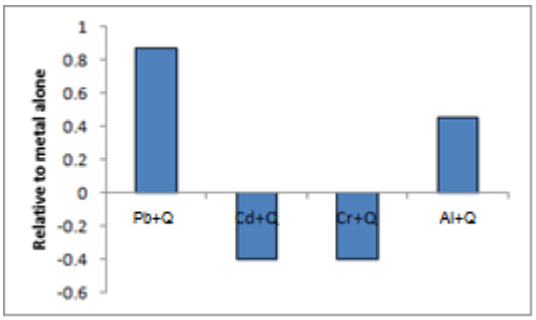


<u>Figure 4.11:</u> Effect of antioxidants catechin, quercetin, gallic acid, caffeic acid, trolox and ascorbic acid on free radicals induced by Pb, Cd, Cr, Al as catalysts of the Fenton reaction. Data is an average of two experiments with three data points each. Standard error of the mean < 0.02%.

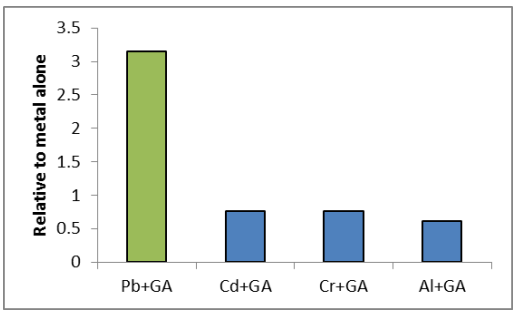
C



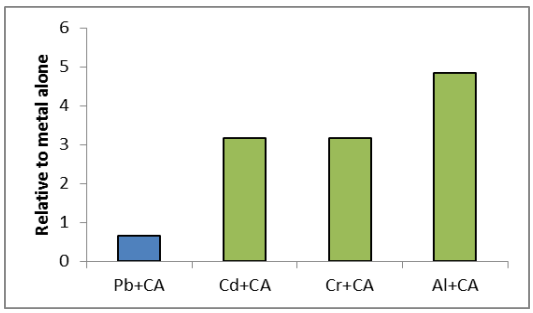
Q



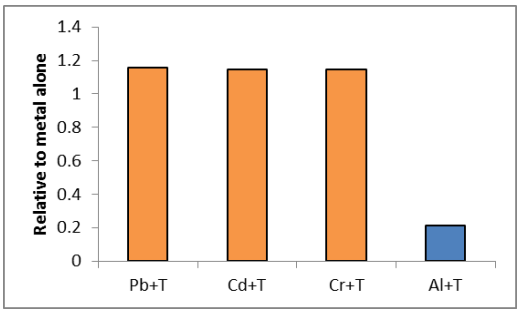
GA



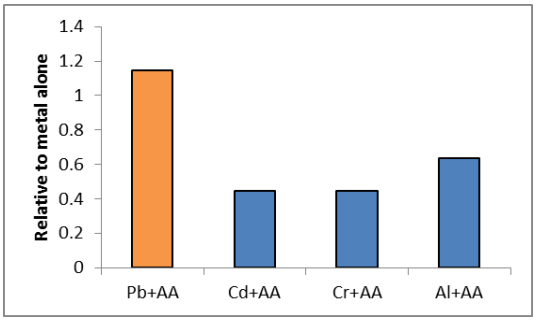
CA



T



AA



<u>Figure 4.12:</u> Effect of antioxidants catechin (C), quercetin (Q), gallic acid (GA), caffeic acid (CA), Trolox (T) and ascorbic acid (AA) on free radicals induced by Pb, Cd, Cr, Al and the Fenton reaction. Observed effects showed no change (orange bars), pro-oxidant effect (green bars) and anti-oxidant (blue bars). Values below 1 indicate an antioxidant effect.

Table 4.4: Effects of antioxidants and metal mediated formation of radicals

	Catechin	Quercetin	Gallic acid	Caffeic acid	Trolox	Ascorbic acid
Pb	**23.70[1]**	0.87	**3.14**	***0.65***	1.15	1.15
Cd	**4.91**	***-0.39[1]***	***0.76***	**3.16**	1.14	***0.44[3]***
Cr	**4.97**	***-0.39[1]***	***0.76***	**3.16**	1.14	***0.44[3]***
Al	**6.38[2]**	***0.46***	**6.12[3]**	**4.85**	***0.21[2]***	*0.63*

Bold indicates a pro-oxidant effect. Bold italic indicates an antioxidant effect. 1-3 indicates interactions with the highest effect.

The proposed mechanism of action is that polyphenols either scavenge free radicals or act as metal chelators thus inhibiting free radical formation.[185] An antioxidant effect was observed with Cr and Cd in combination with quercetin, Al in combination with Trolox and Cd as well as Cr in combination with ascorbic acid.

A limitation of this study was that a single dosage of the antioxidants tested was used and whether an antioxidant has a pro-oxidant effect is dependent on concentration. For example, at low concentrations gallic acid is an antioxidant while at higher concentrations has a pro-oxidant effect. This effect is most likely due to gallic acids strong reducing power.[186]

Tocopherols can also reduce Fe^{3+} to Fe^{2+} and Cu^{2+} to Cu^{+} thus exerting pro-oxidant effects *in vitro*. This Fe-reducing ability of vitamin E and its derivatives was the basis for several colorimetric methods used to detect tocopherols.[177] In a study by El Demerdash[113] *et al.* (2004) it was found that vitamin E and β-carotene reduced Cd induced lipid peroxidation effects. The effects seen in the present study also indicates that vitamin E and its derivatives decrease ROS formation. In this study, Trolox did not effectively reduce Cd catalysed radical formation but Trolox did reduce the effect of Al.

4.8.2 ABILITY OF ANTIOXIDANTS TO PREVENT ERYTHROCYTE OXIDATIVE DAMAGE

Binding of metals to antioxidants would reduce the ability of the metals Pb, Cd, Cr and Al to induce haemolysis via the induction of ROS (Figure 4.13). The antioxidants, gallic acid, catechin and Trolox alone did not induce oxidative damage. In this study only the effect of Pb could be evaluated as Pb was the only metal that induced an increase in ROS formation measured with the DCFH-DA assay. Antioxidants, gallic acid, catechin and Trolox inhibited the oxidative effects of Pb (Figure 4.13). A pro-oxidant effect, due to the synergistic toxic effects was not observed for any metal and antioxidant combinations.

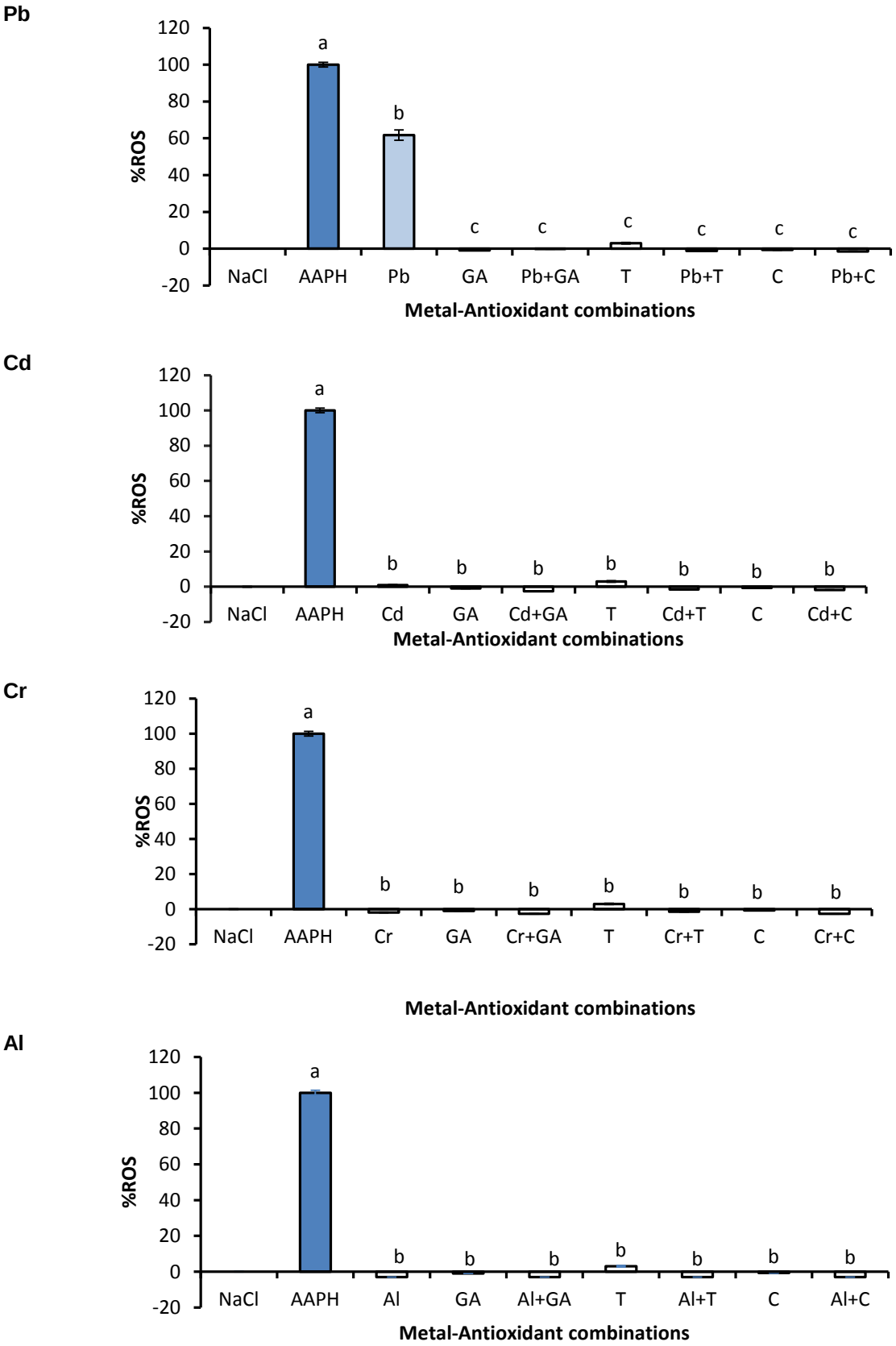


Figure 4.13: The antioxidant effects of antioxidants, gallic acid, catechin and Trolox on heavy metal induced oxidative damage. Controls: NaCl (0% ROS) and AAPH (100% ROS). Antioxidants are gallic acid (GA), catechin (C) and Trolox (T) alone and in combination with Pb, Cd, Cr and Al. Data is an average of three experiments with three data points each. Standard error of the mean < 0.02%. Means with different letters are significantly different, p≤0.001.

Mandal[155] *et al*. (2013), evaluated the protective effect of tea against Cu toxicity in erythrocytes. The results showed that tea (which contains both catechins and gallic acid), prevented morphological alterations to erythrocytes associated with oxidative damage and prevented Cu induced cellular death.[155] The protective effect of HT against Hg induced toxicity in erythrocytes has also been investigated.[181] Using the DCFH-DA assay, increased ROS formation was observed after 4 and 24 hrs exposure to Hg. With the addition of HT, at concentrations as low as 10μM, a decrease in ROS was measured. NAC a thiol containing antioxidant molecule inhibited silver nanoparticles (AgNPs) induced ROS formation in human hepatoma cells.[187] Likewise, in the present study low levels of the antioxidants, gallic acid, catechin and Trolox reduced ROS formed by Pb.

4.9 ABILITY OF ANTIOXIDANTS TO BIND METALS

As shown in Table 4.4 several antioxidants effectively reduced the oxidative effects of Pb, Cd, Cr and Al. It is unknown whether this is due to direct metal binding or the direct quenching of formed radicals.

Traditionally the $AlCl_3$ assay is used to quantify flavonoids. This colorimetric assay is based on the binding of $AlCl_3$ to flavonoids such as catechin and quercetin. In order to evaluate if Pb, Cd and Cr has a similar binding ability, Al was substituted in this assay with Pb, Cd and Cr while Fe(II) and Fe(III) were used as controls. The binding capacity was determined and was reported as the mM metal that had bound 1mM polyphenol (Figures 4.14-4.16 and Table 4.5).

The binding effect of flavonoids, catechin and quercetin was evaluated. Both flavonoids were found to be strong metal binders. From the above results, catechin and quercetin bind with an increasing strength to the metals as follows: catechin: Cr(III) < Al(III) < Pb(II) < Fe(II) < Fe(III) < Cd(II); and quercetrin: Cr(III) <Al(III) < Pb(II) < Fe(III) < Fe(II) <Cd(II). Cd(II) binds the strongest to both catechin and quercetrin.

Flavonoids are found in a variety of fruits and vegetables such as buckwheat, apples and onions. Flavonoid rich drinks are juices, tea and wine.[188] Besides directly scavenging radicals, these polyphenols are also able to bind metals, preventing radical generation and therefore are highly efficient antioxidants.[189-192] Ostrowaska[193] *et al.* (2006) reported that catechins found in tea have the ability to chelate metal ions and prevent their participation in the Fenton and Haber-Weiss reactions, thus decreasing or even preventing the formation of free radicals.[193,194] In this study catechin and quercetin bound all the metals but to varying degrees. In the present study Cd bound the strongest to both flavonoids. Renugadevi and Prabu[195] (2009) evaluated the effect of green tea on Cd induced liver toxicity and found that flavonoids present in green tea effectively quenched free radicals and thus decreased lipid peroxidation.

Three possible mechanisms were identified and these were the direct quenching of radicals, improvement in cellular antioxidant status and the ability of flavonoids to chelate metals. This study confirms that both flavonoids, catechin and quercetin have the ability to chelate heavy metals and protect cells from metal induced oxidative damage.[195] Catechol and gallol groups found in catechin are effective metal chelators and when deprotonated can bind metals. Metals such as Fe(II) and Fe(III) prefer octahedral geometry and can bind up to three catecholate or gallate groups.[196] Likewise, Cd, Cr and Al will also bind. Catechin and quercetin also bind Fe(II) and Fe(III).

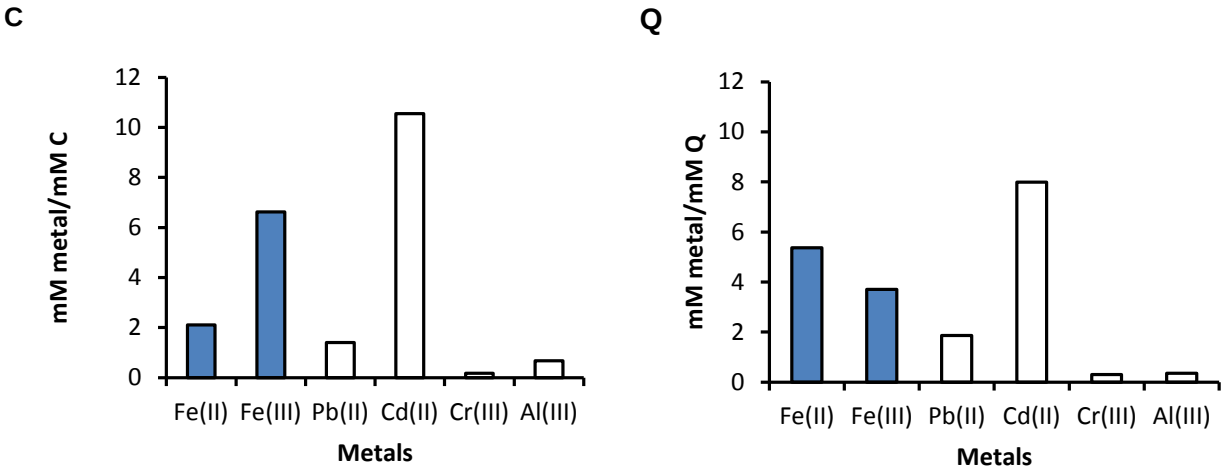


Figure 4.14: Flavonoids: catechin (C) and quercetin (Q) equimolar binding capacity to metal ions, Fe(II) and Fe(III)(controls), Pb(II), Cd(II), Cr(III) and Al(III). Data is an average of three experiments with three data points each.

The ability of each metal to bind phenolic acids, gallic acid and caffeic acid was then determined (Figure 4.15) and was compared to the binding ability of flavonoids catechin and quercetin (Table 4.5). Both gallic acid and caffeic acid were found to have a moderate metal binding capacity. Gallic acid binds Fe(II), Fe(III) and Cd(II) while caffeic acid binds Fe(III). These phenolic acids do not bind Pb, Cr and Al.

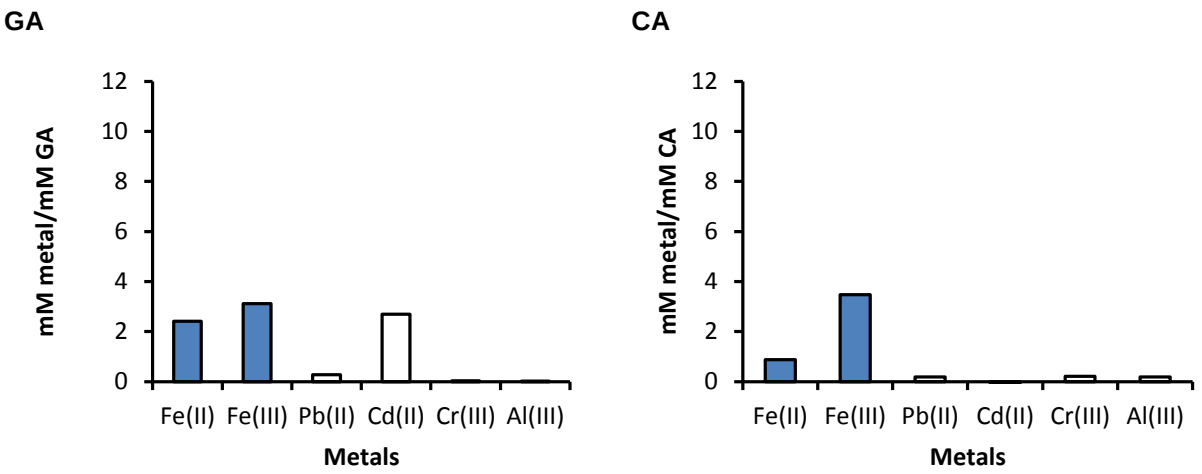


Figure 4.15: Phenolic acid: gallic acid (GA) and caffeic acid (CA) equimolar binding capacity to metal ions, Fe(II) and Fe(III)(controls), Pb(II), Cd(II), Cr(III) and Al(III). Data is an average of three experiments with three data points each.

The ability of Pb, Cd, Cr and Al to bind antioxidant vitamins, Trolox and ascorbic acid was also determined (Figure 4.16). As expected Trolox, a vitamin E analogue does not bind any metals. In contrast, ascorbic acid did bind Fe(III) and showed strong Cd(II) binding ability. Numerous studies have shown that ascorbic acid significantly decreases damage caused by Cd. Ji[197] *et al.* (2012) reported that ascorbic acid alleviated Cd-induced histopathological damage in testes of rats and reduced testicular oedema.[197] Likewise Erdogan[198] *et al.* (2005) reported that oxidative stress induced by Cd caused a decrease in performance of broiler chickens and that dietary supplements of ascorbic acid were useful and reversed these effects and decreased Cd induced lipid peroxidation.[198] Ascorbic acid was also found to reduce Cd absorption and distribution in rats.[199]

Likewise in the present study it was found that ascorbic acid possibly reduces Cd induced ROS formation by direct binding. Ascorbic acid is oxidised to form dehydroascorbic acid in a reversible reaction. During oxidation, it loses an electron to form a radical cation, then loses a second electron to form dehydroascorbic acid. It then typically reacts with ROS.[98] Its oxidised form is relatively unreactive. Surplus ascorbate in the presence of metal ions can initiate and promote free radical reactions, thus making it a potential pro-oxidant.[98]

In this part of the study polyphenols have been shown to bind metals thereby reducing the toxicity of these metals irrespective of whether these metals cause ROS formation, bind GSH or inhibit the antioxidant pathways.

In addition, these findings also confirm that this modified version of the $AlCl_3$ assay can be used to screen antioxidant molecules and possibly mixtures for metal binding abilities.

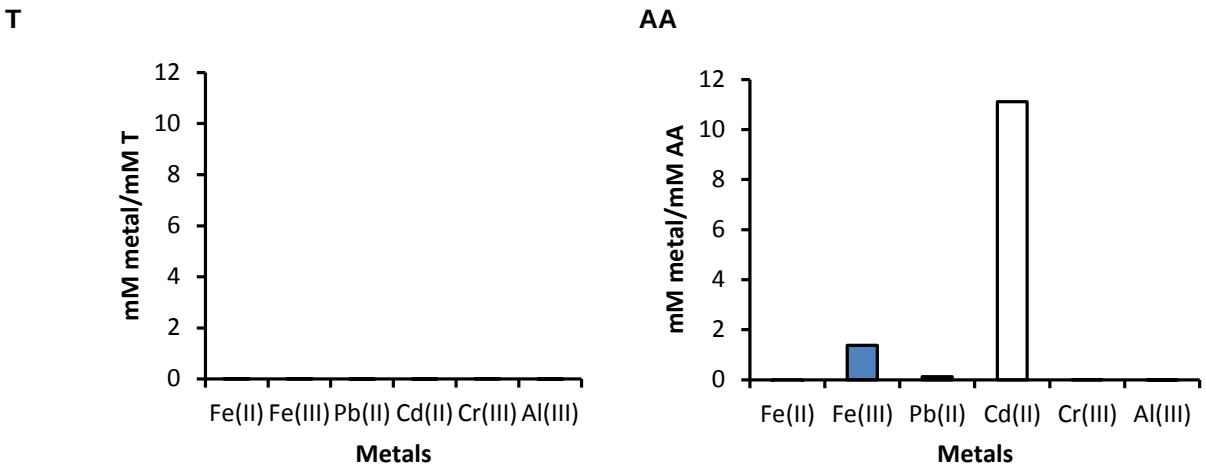


Figure 4.16: Vitamins Trolox (T) and ascorbic acid (AA), equimolar binding capacity to metal ions, Fe(II) and Fe(III)(controls), Pb(II), Cd(II), Cr(III) and Al(III). Data is an average of three experiments with three data points each.

Table 4.5: Summary of Fe, Pb, Cd, Cr and Al equimolar ability to bind antioxidants

	Fe(II)	Fe(III)	Pb(II)	Cd(II)	Cr(III)	Al (III)
Catechin	**5.37**[e]	3.70	1.86	**7.99**[c]	0.30	0.36
Quercetin	2.10	**6.61**[d]	1.40	**10.55**[b]	0.00	0.68
Gallic acid	2.42	3.12	0.27	2.70	0.01	0.01
Caffeic acid	0.88	3.47	0.01	0.01	0.01	0.01
Trolox	0.01	0.01	0.01	0.01	0.01	0.01
Ascorbic acid	0.01	1.38	0.01	**11.11**[a]	0.01	0.01

Letters a-e indicates binding ability from high to low

Of all the antioxidants evaluated gallic acid, catechin and ascorbic acid were the most effective in scavenging the effects of Cd(II). Catechin and quercetin also bound Pb and Al but to a lesser degree than Cd. Of concern is the ability of these antioxidants also to bind Fe(II) and Fe(III). High levels of the antioxidants in the diet such as antioxidants found in health product formulations may reduce Fe levels possibly leading to anaemia, although this effect should be further evaluated in an animal model. Besides antioxidant activity, ascorbic acid also plays an essential role in several metabolic processes which includes the activation of vitamin B and folic acid, the conversion of cholesterol to bile acids and the conversion of the amino acid tryptophan to the neurotransmitter serotonin.[200] Depletion of ascorbic acid levels can result in defects in biological processes, diseases such as scurvy, decreased collagen synthesis which leads to poor wound healing, skin lesions and blood vessel fragility.[98]

4.10 EFFECT OF METAL BINDING TO ANTIOXIDANTS ON THE ANTIOXIDANT ACTIVITY

The metals Pb, Cd, Cr and Al bind to varying degrees to antioxidants, thus preventing GSH depletion and the ability of these metals to catalyse the Fenton reaction. The question is raised whether if with binding the antioxidant activity of the antioxidants is lost. Antioxidant activity can be evaluated using several different assays and these include the TEAC assay. Using the TEAC assay, the ability of antioxidants to scavenge the generated $ABTS^{\cdot+}$ radical was evaluated. Activity is expressed as relative to that measured for Trolox.[154] In this study the TEAC assay was used to determine the antioxidant capacity of mixtures of polyphenols and metals. These results are presented in Figures 4.17, 4.18 and 4.19.

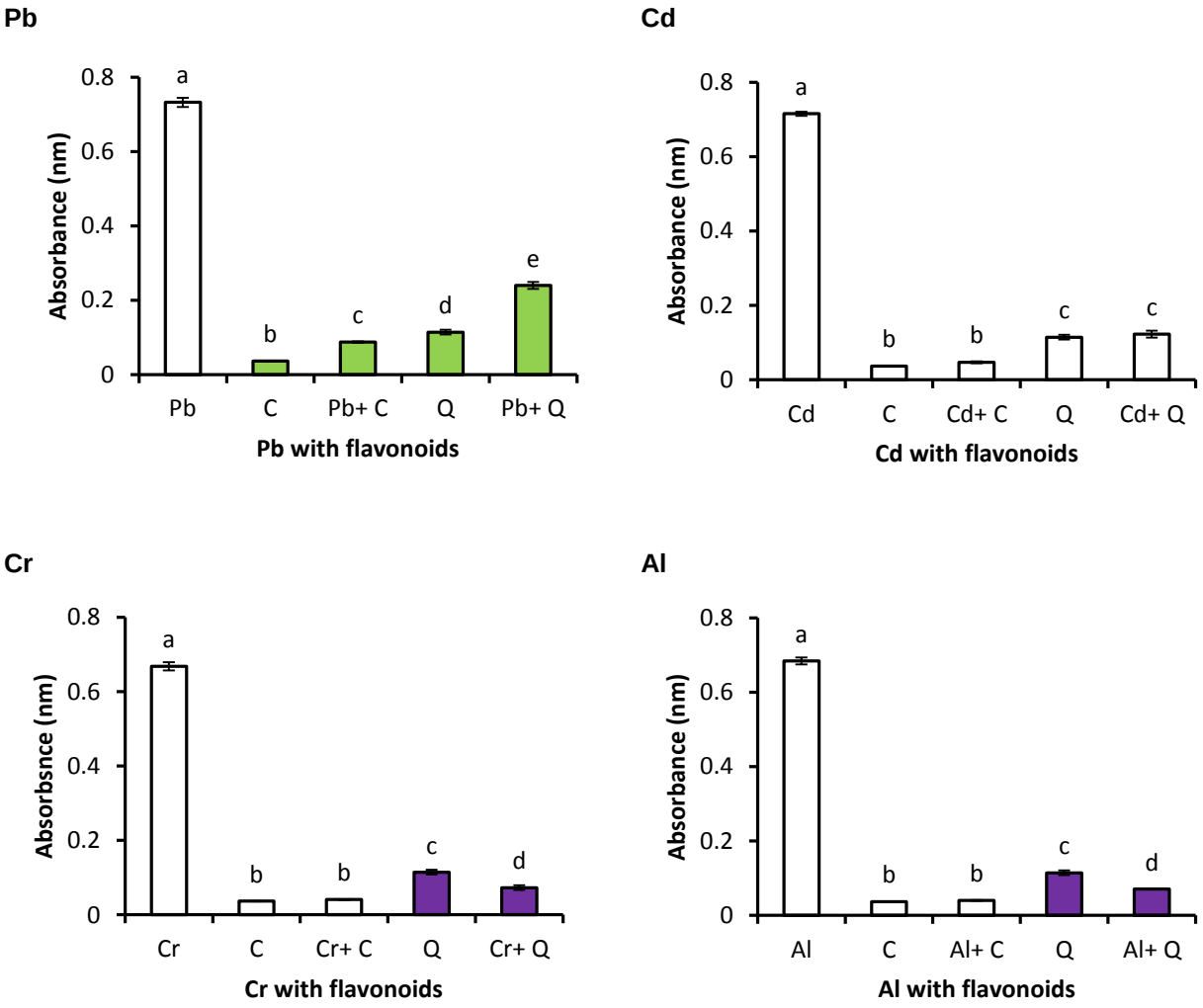
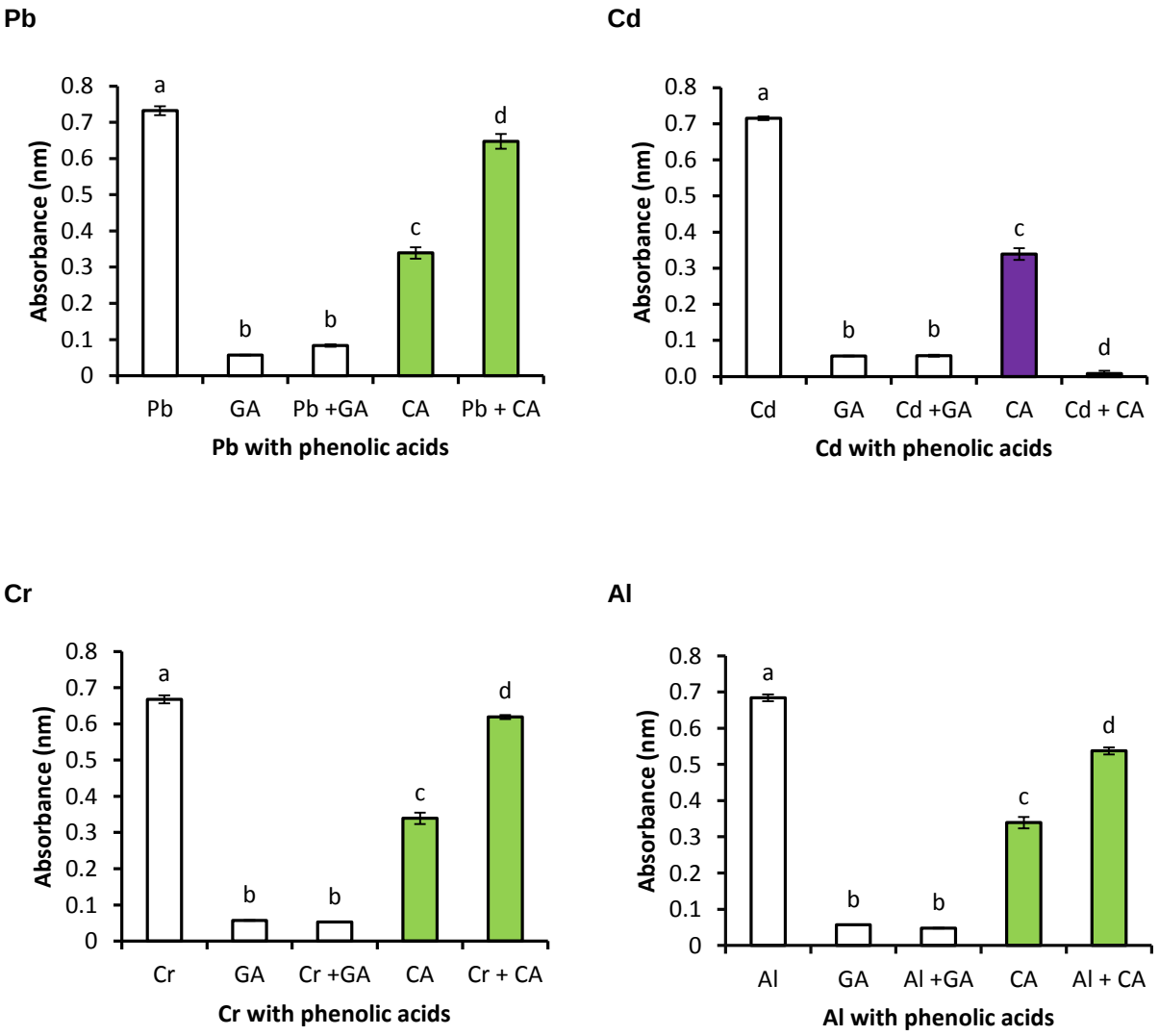


Figure 4.17: Antioxidant activity of flavonoids, catechin and quercetin in the presence of metals Pb, Cd, Cr and Al. Data is an average of three experiments with three data points each. Standard error of the mean < 0.02%. Means with different letters are significantly different, p≤0.001. Green indicates a loss of antioxidant activity, purple an enhancement of activity.

All metals do not react with the $ABTS^{\cdot+}$ radical. The flavonoids catechin and quercetin (Figure 4.17) effectively reduces radical formation by 94.5% and 82.8% respectively. Catechin and quercetin binding to Pb caused a decrease in the measured antioxidant activity of these flavonoids. Cd binding to catechin and quercetin (Table 4.5) did not alter the antioxidant activity of these flavonoids. Cr and Al had no effect on the antioxidant activity of catechin but did enhance the antioxidant activity of quercetin.



<u>Figure 4.18:</u> Antioxidant activity of phenolic acids, gallic acid and caffeic acid in the presence of metals Pb, Cd, Cr and Al. Data is an average of three experiments with three data points each. Standard error of the mean < 0.02%. Means with different letters are significantly different, p≤0.001. Green indicates a loss of antioxidant activity, purple an increase in activity.

The interaction between the metals, Pb, Cd, Cr and Al with gallic acid and caffeic acid was then evaluated. Measured antioxidant activity of gallic and caffeic acid was 92.20% and

53.56% respectively. Pb, Cd, Cr and Al had no effect on the antioxidant activity of gallic acid. Pb, Cr and Al reduced the antioxidant activity of caffeic acid. The mechanism involved is not metal binding (Table 4.5). Cd binding to caffeic acid caused an enhancement of the antioxidant activity of caffeic acid although the mechanism involved is unknown.

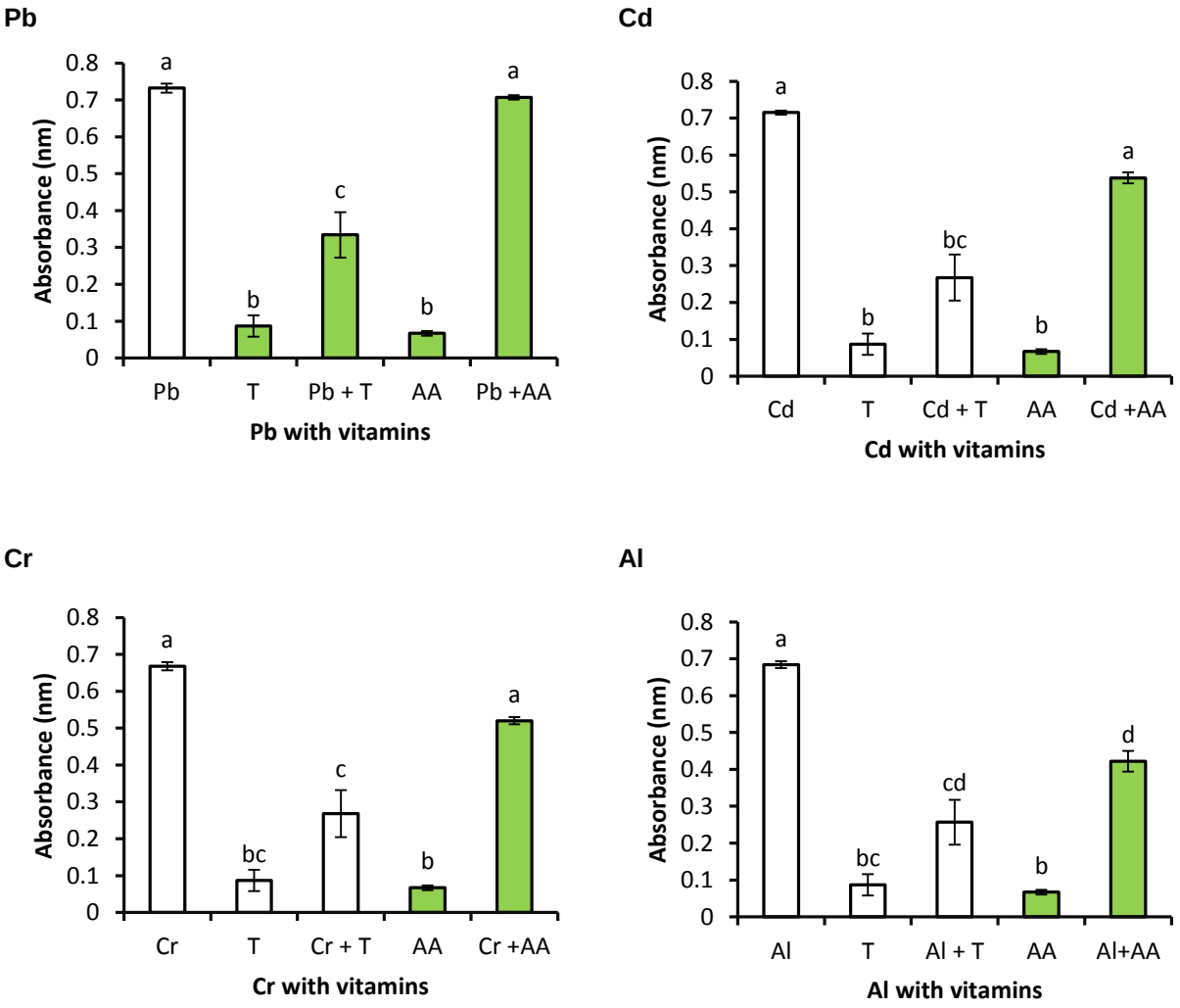


Figure 4.19: Antioxidant activity of vitamins, Trolox and ascorbic acid in the presence of metals Pb, Cd, Cr and Al. Data is an average of three experiments with three data points each. Standard error of the mean < 0.02%. Means with different letters are significantly different, p≤0.001. Green indicates a loss of antioxidant activity, purple an increase in activity.

Antioxidant vitamins, ascorbic acid and Trolox reduced radical formation by 88.08% and 90.80% respectively. The antioxidant activity of Trolox when mixed with metals was reduced, it was only statistically significant for the combination of Pb and Trolox. In contrast, all metals reduced the antioxidant activity of ascorbic acid and the greatest effect was observed for

ascorbic acid in combination with Pb. The loss of antioxidant activity between ascorbic acid and Cd was due to binding (Table 4.6). The mechanism whereby Pb, Cr and Al reduces activity is unknown.

For each combination, the fold change in antioxidant activity was calculated to determine which metals to the greatest degree compromised the activity of antioxidants (Table 4.6).

The greatest effect was observed for Pb in combination with ascorbic acid while the lowest effect was for quercetin in combination with Cr and Al. All metals evaluated had a major impact on the antioxidant activity of ascorbic acid. This is of major concern as ascorbic acid is a major vitamin in fresh fruits and vegetables and loss of activity may result in depletion and consequently several biochemical pathways may become compromised.

Table 4.6: The fold effect of heavy metals Pb, Cd, Cr and Al on antioxidant activity of antioxidants

	Flavonoids		Phenolic acids		Vitamins	
Metal	**Catechin**	**Quercetin**	**Gallic acid**	**Caffeic acid**	**Trolox**	**Ascorbic acid**
Pb	2.4	2.1	1.5	1.9	**3.9**[e]	**10.5**[a]
Cd	1.3	1.1	1	***0.02***[e]	3.1	**8.0**[b]
Cr	1.1	***0.6***[f]	0.9	1.6	3.1	**7.8**[c]
Al	1.1	***0.6***[f]	0.8	1.4	3.0	**6.3**[d]

Letters, a-e indicates loss of antioxidant activity, from high to low. Letter e-f indicates enhanced activity, from high to low.

4.11 ABILITY OF ANTIOXIDANTS TO PREVENT METAL - GSH BINDING

In addition to antioxidants preventing ROS formation, metal antioxidant binding may also cause the depletion of GSH (Figure 4.20 and Table 4.7). In Section 4.6.1 it was found that Cd, Cr and Al bound GSH and reduced the measured GSH levels. For Pb only a 20% decrease in the GSH levels was measured. In the presence of the antioxidants, gallic acid, Trolox and catechin, Pb levels were further reduced. While in contrast, a small protective effect was observed for Cd and Cr with Trolox and catechin (Table 4.7). Antioxidants had no effect on Al binding of GSH. In Table 4.7, catechin protected GSH from Cr binding while Trolox reduced the binding ability of Cr, Cd and Pb.

In this experiment 0.25mM of each antioxidant, 0.25mM GSH and 13mM Pb, 18mM Cd, 30mM Cr and 63mM Al were combined. In order to better understand the mechanisms

involved, metal levels should be reduced and a dosage study should be undertaken as the type of reaction may be a direct stoichiometric interaction between the antioxidants, metal and GSH.

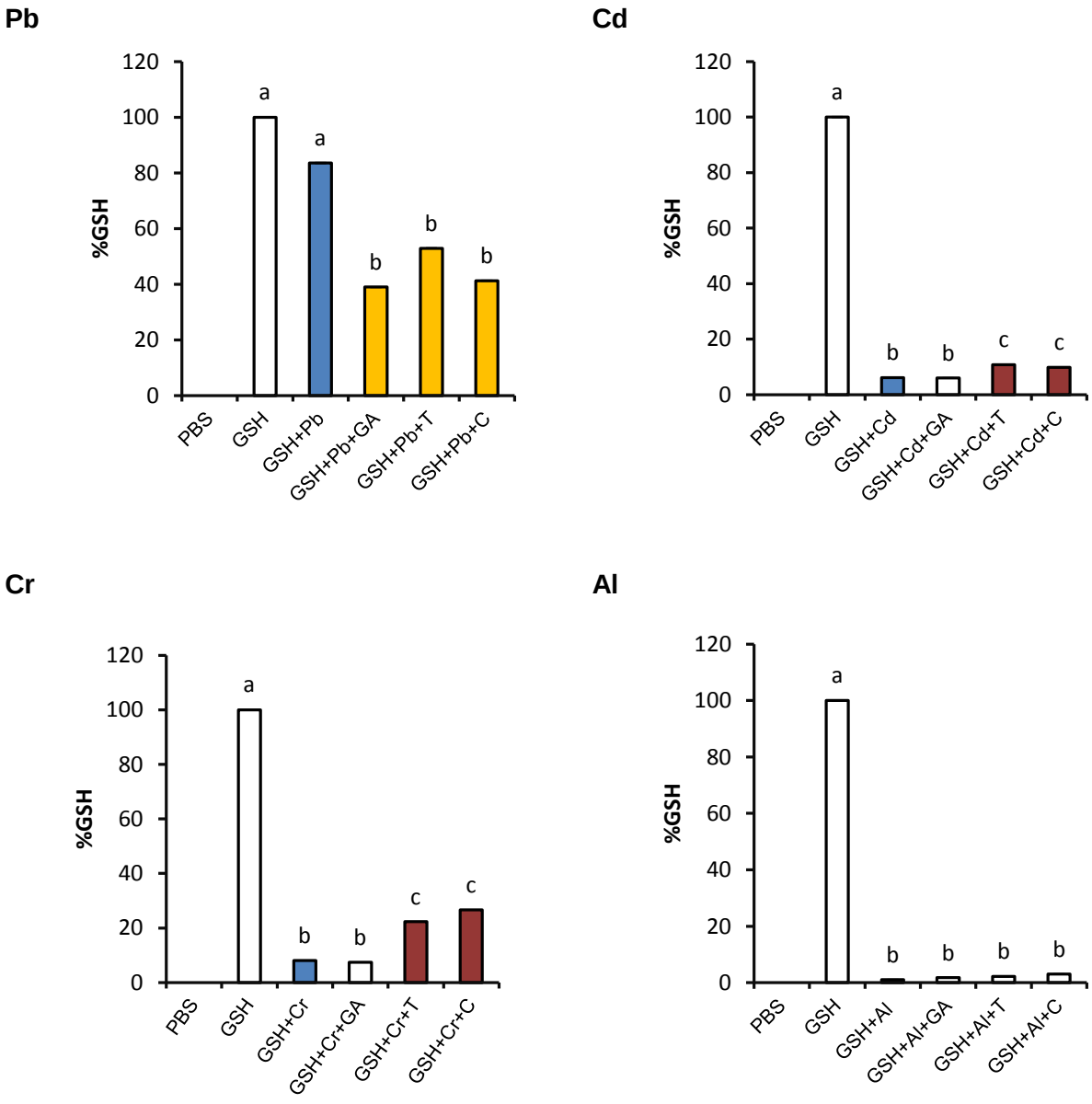


Figure 4.20: The ability of a 0.25mM concentration of antioxidants gallic acid (GA), Trolox (T), catechin (C) to prevent A) 13mM Pb, B) 18mM Cd, C) 30mM Cr and D) 63mM Al binding to 0.25mM GSH. Data is an average of three experiments with three data points each. Standard error of the mean < 0.02%. Means with different letters are significantly different, p≤0.001. Blue indicated GSH with the metal, orange indicates partial depletion of GSH, whereas red indicates complete depletion of GSH.

Table 4.7: Summary the effect of antioxidants on metal induced GSH depletion

Metal	Catechin	Gallic acid	Trolox
Pb	**2.02[b]**	**2.13[a]**	**1.57[c]**
Cd	1.70	NS	***1.75[f]***
Cr	***3.31[d]***	NS	***2.76[e]***
Al	NS	NS	NS

Letters a-c indicates binding ability, increase in binding, from high to low. Letters d-f indicates protection against metal GSH binding effects from high to low. NS indicates, no statistical difference.

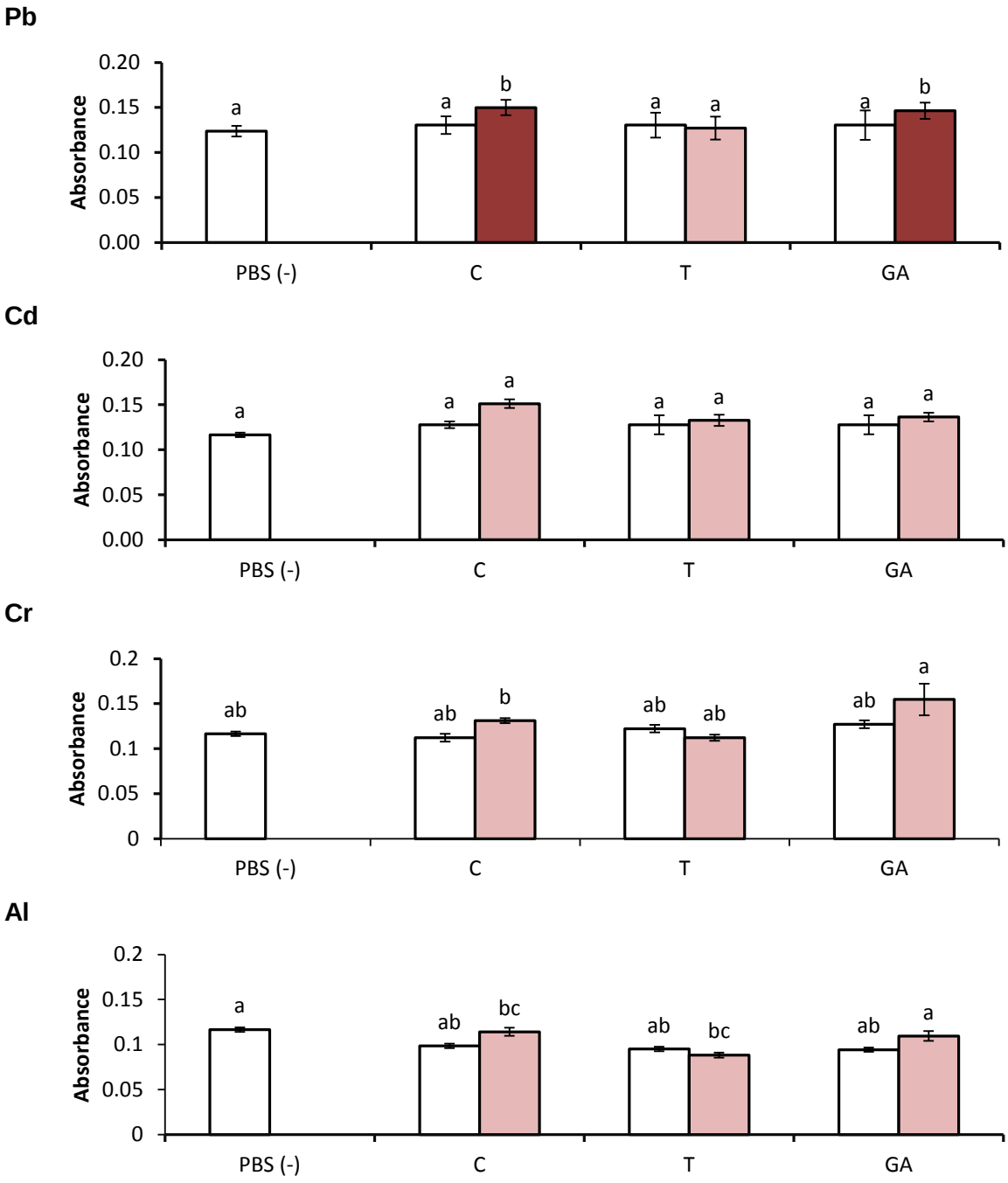
4.12 EFFECT OF ANTIOXIDANTS ON ERYTHROCYTE GSH LEVELS

In contrast to previous reported findings in the present study, it was found that incubation of erythrocytes with heavy metals did not cause a depletion of GSH levels. Pb caused a significant increase in GSH levels and this was attributed to the ability of Pb to inhibit the antioxidant enzyme GPx. Cd, Cr and Al had no effect on GSH levels. Therefore, if the metals, especially Pb adversely affect the function of GPx, then the addition of antioxidants will lower GSH levels to normal.

The effect of antioxidants, catechin, Trolox and gallic acid on erythrocyte GSH levels was determined following exposure to Pb, Cd, Cr and Al (Figure 4.21, Table 4.8). Levels were compared to metals alone. For Pb, catechin and gallic acid caused a statistical 1.2 and 1.1 fold increase in GSH levels. No change in GSH levels was observed for Cd, Cr or Al. As for the inhibition of metal-GSH binding, by antioxidants in contrast to low concentrations of antioxidants required to inhibit ROS formation to inhibit the effects of metals on antioxidant pathways may require higher concentrations or pre-incubation with antioxidants prior to the addition of metals.

Supplementation with curcumin and vitamin E reduced diazinon-induced oxidative damage in rat livers and erythrocytes and modulated GSH levels.[201,202] Administration of the antioxidant selenium prior to exposure of male rats to Pb caused a noticeable prophylactic action by increasing the activities of SOD and GR and increasing the GSH content.[202] The effect of *Emblica officinalis* (Amla) on metal–induced lipid peroxidation in human erythrocytes was investigated. Antioxidants found in Amla increased the levels of GSH, which the authors attributed to the increased specific activity of GPx.[203] However, erythrocytes exposed to toxic

levels of Pb, have increased GSH levels and in this study this is attributed to the direct inhibition by Pb of the GSH-GSSG antioxidant pathway.



<u>Figure 4.21:</u> Ability of 0.25mM antioxidants (catechin (C), Trolox (T) and gallic acid (GA)) to prevent erythrocyte GSH depletion by 13mM Pb, 18mM Cd, 30mM Cr and 63mM Al. Data is an average of three experiments with three data points each. Standard error of the mean < 0.02%. Means with different letters are significantly different, p≤0.001. Solid filled indicates a significant increase in GSH compared to metal alone.

Table 4.8: Effect of antioxidants on GSH levels of metal exposed erythrocytes

	Catechin	Gallic acid	Trolox
Pb	**1.2**	**1.1**	NS
Cd	NS	NS	NS
Cr	NS	NS	NS
Al	NS	NS	NS

NS indicates no statistical difference.

In conclusion several aspects of antioxidant protection against the adverse effects of Pb, Cd, Cr and Al as radical scavengers and as molecules that prevent metal GSH binding. In general antioxidants had a beneficial effect although effects were variable. Although this is an exploratory study, it highlights the importance of a mixed diet of fruit and vegetables, containing different types and concentrations of antioxidants. The molecules may act synergistically or have different mechanisms or targets thereby protecting cells and tissue against oxidative damage.

CHAPTER 5: CONCLUSION

5.1 RATIONALE FOR THE STUDY

Besides environmental exposure to metals, cigarette smoke either primary or secondary contributes to exposure. This exposure in South Africa as well as in the rest of the world is increasing.[1] Exposure to heavy metals such as Pb, Cd, Cr and Al can cause heavy metal toxicity and this includes depletion of antioxidant pathways via the depletion of antioxidant elements such as GSH, and inhibition of antioxidant enzymes. A diet low in endogenous antioxidants such as polyphenols and antioxidant enzymes acerbates this toxic effect. Supplementation with dietary antioxidants, if positive would be a cost effective, relatively simple method in helping to decrease the toxic effects of heavy metals exposure. Therefore, the aim of this study was twofold, namely to determine the toxicity of metals commonly found in cigarette smoke using an *ex vivo* model and then to determine if antioxidants that are bioavailable can reduce toxicity.

5.2 SUMMARY OF RESULTS

5.2.1 METAL TOXICITY

Pb was found to be the most toxic metal investigated in this study causing H50% at 16.00mM which was associated with echinocyte, type III formation. Pb was compared to Cd, Cr and Al and was a poor catalyst of the Fenton reaction. However, following exposure of erythrocytes to Pb, increased ROS formation occurred. Pb did not bind GSH but in erythrocytes increasing concentrations of Pb resulted in an increase in GSH levels. This implies that Pb does affect the erythrocyte oxidative pathways but this is not as a catalyst of the Fenton reaction or due to GSH binding. The observed effect/s may be due to Pb inhibiting antioxidant enzyme activity, causing an increase in GSH levels or the effect of Pb on ALAD as described in Section 2.3.1.[28]

Cd induced H50% at 33.83mM and the degree of haemolysis and the associated changes in erythrocyte morphology (formation of spherocytes) was similar to that induced by the oxidant AAPH. Cd catalysed the Fenton reaction and bound GSH, however in erythrocytes Cd did not cause an increase in ROS or alter GSH levels. This observed effect in erythrocytes may be due to the high catalase activity found in erythrocytes or due to the lack of endogenous H_2O_2 required for the generation of $OH^{\bullet}$ radicals. Alternatively, Cd may directly target cellular

components such as the cell membrane causing changes in morphology and fluidity leading to haemolysis or cause energy depletion.

At 47.83mM Cr causes H50% and induced echinocyte type III formation. Cr catalysed the Fenton reaction and bound GSH in a manner similar to Cd. Likewise, Cr did not cause an increase in ROS formation but did cause changes in GSH levels. The higher Cr concentrations required for haemolysis and echinocyte type III formation implies that Cr may be slightly less toxic than Cd. The mechanism of action may be similar to Cd.

Of all the metals investigated Al, was the least toxic, with H50% occurring at 81.26mM associated with formation of echinocytes, type I. Al was a poor catalyst of the Fenton reaction but did bind GSH. In *ex vivo* erythrocytes, Al did not induce ROS formation or cause changes in GSH levels. Al has been implicated in the development of neurological disease such as Alzheimer's disease, Amyotrophic Lateral Sclerosis (ALS), dementia and Parkinson's disease.[204] Observed effects may be due to differences in the cell types used where neurons may be more sensitive to the oxidative effects of Al.

5.2.2 PROTECTION: METALS AND ANTIOXIDANTS

Pb was the most toxic metal investigated and caused haemolysis and ROS induced oxidative damage. Pb was a poor catalyst of the Fenton reaction and did not bind GSH. Antioxidants did cause some inhibition of haemolysis. Catechin, gallic acid and Trolox effectively reduced the oxidative effects of Pb and GSH levels remained increased in the presence of catechin and gallic acid. Catechin, gallic acid and Trolox appeared to bind GSH and reduce the amount of free GSH that could be quantified.

Cd caused haemolysis, catalysed the Fenton reaction and bound GSH although these effects did not cause an increase in ROS formation. Antioxidants did cause some inhibition of haemolysis and antioxidants, quercetin and ascorbic acid inhibited the Fenton reaction and bound Cd. This metal antioxidant interaction caused a loss in the antioxidant activity of ascorbic acid but enhanced the activity of caffeic acid. Trolox reduced, Cd–GSH binding.

Similar to Cd, Cr caused haemolysis however this was not associated with ROS formation. Cr did catalyse the Fenton reaction and bind GSH. Catechin and gallic acid reduced haemolysis. In the Fenton reaction, quercetin and ascorbic acid scavenged hydroxyl radicals. Cr decreased the antioxidant activity of ascorbic acid, and enhanced the antioxidant activity of quercetin. Catechin and Trolox reduced the ability of Cr to bind GSH.

Al was the least toxic of all the metals evaluated. Al did cause haemolysis although this was not associated with ROS formation or changes in GSH levels. Al was a poor catalyst of the Fenton reaction but did bind GSH. Catechin, gallic acid and Trolox reduced haemolysis. Trolox inhibited Al catalysis of the Fenton reaction. Al had no effect on the antioxidant activity of catechin, gallic acid and Trolox, although metal antioxidant interactions enhanced the activity of quercetin and caused a loss in the antioxidant activity of ascorbic acid. Antioxidants did not alter erythrocyte GSH levels.

In summary, some antioxidants especially catechin, gallic acid, Trolox and ascorbic acid prevented metal induced cellular damage. The observed effects may be related to direct radical scavenging, GSH protection against metal binding or may be due to unknown membrane effects especially considering the effect of Trolox. Of concern is the adverse effect of these metals on the bioactivity of ascorbic acid.

5.3 IMPLICATIONS FOR THE STUDY

Ascorbic acid supplementation ameliorates the adverse effects of nicotine on placental haemodynamics and histology in non-human primates. Lo *et al.* (2015)[205] suggest that supplementation of ascorbic acid decreased the harmful effects of prenatal nicotine exposure and is suggestive of limiting some of the adverse effects associated with smoking during pregnancy.[205] A randomised clinical trial showed that offspring of women that smoke and who had received ascorbic acid supplementation had significantly decreased wheezing up to the age of 1 year. Both studies show that ascorbic acid supplementation may be a cost effective and simple approach to decrease the effects of smoking in pregnant women on the pulmonary function of neonates.[206]

This study using erythrocytes clearly showed that Ascorbic acid effectively reduced metal toxicity. This implies that this model can be used to rapidly identify additional antioxidants with bioactivity or be used to evaluate and optimize formulations that can reduce the adverse effects of smoking, especially on the pulmonary function of neonates. This effect may also include individuals exposed to secondary smoke and high levels of air pollution.

In a randomised, double blind, placebo-controlled, cross-over trial conducted by Bo *et al,.* (2013)[207] stated that the anti-inflammatory and antioxidant effects of the antioxidant resveratrol in healthy smokers was evaluated. Resveratrol was found to have both antioxidant and anti-inflammatory effects and supplementation may reduce cardiovascular disease risk seen in healthy smokers.[207] Polyphenols also have anti-inflammatory and anticancer activity and supplementation may prevent the development of COPD and lung cancer.[178, 207] The role of

polyphenols as anti-inflammatory and anti-cancer agents has to do with their chemical makeup and their effect on molecular pathways. One molecular mechanism that has been identified in their anti-inflammatory effect is the inhibition of enzymes related to inflammation. [178, 207]

Oxidative stress plays a major role in the development of chronic pulmonary complications. Panahi *et al.* (2016)[208] conducted a randomised controlled trial, which found a beneficial effect of curcuminoids-piperine combinations on systemic oxidative stress, clinical symptoms and quality of life in subjects with chronic pulmonary complications caused by sulfur mustard (a cytotoxic warfare agent).[208] The study concluded that these phytochemical formulations such as the ones used in this study can be used as safe adjuncts in patients suffering from pulmonary complications caused by sulphur mustard and who are receiving standard treatment.[208] In the present study, ascorbic acid prevented metal induced cellular damage by inhibiting oxidative damage caused by the Fenton reaction, by scavenging free radicals formed by the Fenton reaction and by binding to heavy metals.

5.4 LIMITATIONS OF THE STUDY

The concentrations per cigarette of each metal are 1.2µg (0.006µmol) Pb, 0.5-1.5µg (0.0044-0.013µmol) Cd, 0.002-0.5µg (0.00004-0.0096µmol) Cr, 699-1200µg (25.91-44.47µmol) Al (Bernhard[4] et al. (2005)). For example, if 20 cigarettes are smoked per day this translates to 24µg (0.12µmol) Pb, 30µg (0.26µmol) Cd, 10µg (0.192µmol) Cr and 24000µg (889.4µmol) Al. The blood volume of an individual weighing 70kg is 5.25L, therefore blood concentrations with 100% absorption and where no metabolism and excretion occurs, the total dosage/day is 0.022µM Pb, 0.050µM Cd, 0.037µM Cr and 169.40µM Al. Therefore, metal concentrations used in this study are too high to reflect the effects *in vivo.* However, this study does identify possible modes of action.

The concentration of metals (Pb, Cd, Cr and Al) were not the same since concentrations were chosen that caused approximately H50% and provides little information on equimolar effects. However, the concentrations do to some degree reflect the different concentrations of each metal found in cigarette smoke where Pb, Cd and Cr is in the same range and Al is much higher. Metals in cigarettes are found as a mixture and this study did not investigate the possible synergistic toxic effects between metals.

This was an exploratory study to develop models for rapid evaluation of toxicity (erythrocyte haemolysis, DCFH-DA assay and the measurement of GSH levels).[209] The exploratory nature of this study allows it to provide insights into the toxicity of the heavy metals and the protection

of antioxidants, however a more comprehensive study must be conducted to acquire further results. Cigarettes affect the mucosa of the bronchial tree which consists of the larynx, the trachea, primary bronchus, secondary bronchus, tertiary bronchus, bronchioles, alveolar ducts with alveoli and the air blood barrier of the lungs making the latter more permeable.[210] Therefore, due to the oxygen carrying properties of erythrocytes these cells are often the first targets of exposure following absorption of the components of cigarette smoke due to the close proximity of blood vessels to the alveoli.[211,212]

Carcinogenesis is associated with alterations to DNA structure such as DNA adduct formation.[213] If these adducts are not excised by the DNA repair enzymes, permanent mutations in the DNA can occur. If these mutations occur in critical regions of an oncogene such as Kirsten rat sarcomas (KRA) or a tumour suppressor gene such as tumour protein 53 (TP53), it results in a loss of normal growth control mechanisms and leads to the development of cancer.[213] A limitation in using human erythrocytes is that these cells do not contain organelles and DNA, thus the effect of cigarette smoke and its toxins on nucleated cells of the bronchial tree and the lungs, and specific sites of tumour development cannot be investigated. For the same reasons this study provides no information on the effect of these metals on the development of COPDs like emphysema.

For all assays a dosage effect was not determined such as in the DCFH-DA assay. In addition, it was found that to form free radicals, H_2O_2 is required as the substrate of the Fenton reaction as shown in Section 2.4.4. Therefore, if H_2O_2 was added as a substrate in the DCFH-DA assay, metals, Cd and Cr may have cellular oxidative effects due to radical formation. Erythrocytes have high levels of catalase and consequently it may be necessary to mix the metals with H_2O_2 prior to exposure to erythrocytes.[123,214]

Dietary polyphenols have been shown to reduce the risk for cancer. A limitation of this study due to the cell type used, did not allow for this aspect of antioxidant action to be investigated.

5.5 FUTURE PERSPECTIVES

The erythrocyte model can be used to further investigate the effects of other metals that are environmental pollutants alone and in combination. Metals can be chosen that have an impact on the health and well-being of the South African population. [215]

This study identified that the oxidative effects of Pb caused haemolysis, however for Al, Cr and Cd this was not the mode of action. Another possible mechanism is energy depletion which is associated with changes in Ca^{2+} channel functioning. Loss of K^+ through these

channels leads to cell shrinkage, whereas the exit of K^+ and entry of Na^+ leads to swelling. Ca^{2+} entry into cells also leads to alterations of the membrane phospholipids and induces morphological changes.[216] Excessive swelling may lead to cell membrane dysfunction leading to haemolysis.[217,218]

Further studies can also include the effect of these metals on organ specific cell lines such as the immortalised HBE1 (human bronchial epithelial cell line), the non-invasive lung cancer cell line, the CL1-0 (lung adenocarcinoma cell line), and its more metastatic clone CL1-5 (lung adenocarcinoma cell line). Thai[219] *et al*. (2013) investigated the effect of cigarette smoke on a novel long non-coding Ribonucleic acid (RNA), Smoke and Cancer Associated LncRNA 1 (SCAL1) cell line. A549 (adenocarcinomic human alveolar basal epithelial cells) were used in a study by Checa[220] *et al*. (2016) to investigate if cigarette smoke increases the expression of profibrotic molecules in alveolar epithelial cells.[219,220] Many of these cell lines are cancer cell lines and do not necessarily reflect the response of normal epithelium of the bronchi and lungs. Primary cultures or an animal based study can be used to address these limitations. According to Churg *et al*. (2008), the C57BL/6 mouse model is the most appropriate model when investigating lung complications associated with cigarette smoke.[221]

In addition, the effects of metal mixtures as found in cigarettes can also be investigated using both *in vitro* and *in vivo* models to investigate their synergistic effects. The effect on RNA and protein expression as well as cellular metabolic activity compared to control cells can be determined. The effect of these metals alone and in combination on the expression of cancer associated genes such as tumour suppressor genes is also an important aspect that can be further researched. Immunohistochemistry, protein blotting, *in situ* hybridisation and quantitative reverse transcription polymerase chain reaction (RT-PCR) can be used to examine changes in gene expression in cell lines and animals exposed to each metal alone and in combination.

Polyphenols were found to reduce the toxic effects of Pb, Cd, Cr and Al. In order to reduce the toxic effect of Pb, Cd, Cr and Al it is necessary to identify polyphenols that do not undergo neutral pH degradation and that are bioavailable.[222] Likewise, the effect of fruit and/or vegetable water extracts on markers of Pb mediated oxidative damage can also be further investigated in a rat model.

Kasperczyk[36] *et al*. (2015) showed that Pb caused MDA and protein carbonyl levels that were significantly increased, whereas proteins and protein sulfhydryls were significantly decreased in Pb exposed workers.[36] Likewise, using an animal model the effects of Cd, Cr and Al on lipid peroxidation, protein carbonylation and GSH can also be determined in blood and lung tissue.

The effects of polyphenols as well as fruits and vegetables rich in polyphenols can then be determined. This can lead to dietary intervention studies.

A concern identified in this study was that ascorbic acid can bind metals. Khand[223] *et al*. (2014) reported that smoking caused a significant decrease in plasma ascorbic acid and α-tocopherol levels compared to non-smokers.[223] It has also been found that the incidence of periodontal disease in smokers is much higher than that of non-smokers, and this was associated with lower plasma ascorbic acid levels in smokers.[224] Furthermore ascorbic acid is also an important co-factor in many important biochemical processes such as collagen biosynthesis and disruption of these pathways can lead to non-lung associated disease such as periodontal disease.

CHAPTER 6: REFERENCES

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