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Investigating the effect of the heavy metals cadmium, chromium and lead, alone and in combination on an endothelial cell line

Submitted in partial fulfilment for the degree Magister Scientiae in Anatomy with specialization in Human Cell Biology

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Declaration

I, **Leigh-Ann van Strijp**, declare that this **dissertation** entitled: **"Investigating the effect of the heavy metals cadmium, chromium and lead, alone and in combination on an endothelial cell line"** which I hereby submit for the degree **Magister Scientiae in Anatomy with specialization in Human Cell Biology** at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

I understand what plagiarism is and am aware of the University's policy in this regard.

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I have not used work previously produced by another student or any other person to hand in as my own.

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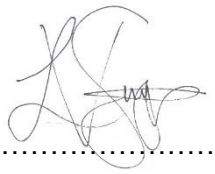
The author, **Leigh-Ann van Strijp**, has obtained for the research described in this work, the applicable research ethics approval. The author declares that they have observed the ethical standards required in terms of the University of Pretoria's code of ethics for researchers and the policy guidelines for responsible research.



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Summary

Heavy metals are natural elements characterized by their relatively large atomic mass as well as their high density. Heavy metal poisoning has occurred in numerous countries in the past, with the United States having the highest prevalence. Heavy metals can also be introduced into the ecosystem by the mining of heavy metals from deep within the earth's crust thereby exposing them into air and water systems.

Another common source of heavy metal exposure is cigarette smoke. Cigarette smoke has been shown to have carcinogenic, toxic and genotoxic properties. Although all the toxic elements in cigarette smoking have not yet been identified, several heavy metals have been found to contribute to the pathophysiological consequences associated with smoking. A study conducted by Yaprak *et al*., in 2019 indicated that cadmium, lead and chromium are the most abundant metals found in cigarette smoke. The current study therefore focused on cadmium, lead and chromium, alone and as part of metal mixtures to determine the role of these heavy metals on endothelial cells. The EA.hy926 endothelial cell line was exposed to different concentrations of each of these metal and their combinations and analysed at different time points to determine the effect of the heavy metals on endothelial cell function. The cytotoxicity of the metals was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Crystal Violet assays, reactive oxygen species productions was studied using the 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) assay, and cell viability was determined using flow cytometry. Finally, morphological changes caused by these metals was studied using scanning electron microscopy.

Exposure of endothelial cells to cadmium, lead and chromium alone and in combination at three different concentrations (X0.1, X1 and X2) showed no significant cytotoxicity as indicated by the MTT and Crystal Violet assay results. At 24 h exposure, cadmium alone showed an increase in percentage free radical formation whereas lead alone showed the greatest percentage radical formation for the X1 concentration group. At 48 h exposure, chromium alone as well as the triple combination group showed an increase in the percentage radical formation between 0.1 and X2 concentrations. Cadmium caused the highest percentage radical formation in the X1 concentration group and lead at the X2 concentration group. After 72 h, both the cadmium and lead as single metals showed a gradual increase in percentage radical formation between the 0.1 and X2 concentrations, with cadmium showing the highest increase in the X1 concentration. Flow cytometric analyses with the Annexin V and the Propidium Iodide assay, showed an increase in early apoptotic and necrotic cells with higher concentrations of the lead and chromium

combination as well as in the triple combination group. Increased necrosis was also evident in the cadmium and chromium combination, the lead and chromium as well as the triple combination groups. Morphological changes were also seen with scanning electron microscopy, with almost all of the X2 concentrations of metals showing either damage to the cell membrane, cell blebs present or a combination of the two. As endothelial cells have a protective function in the human body, cigarette smoke diminishes its protective ability through both disruption of cellular processes and morphology. Extensive research has been done on pulmonary endothelial cells and the link that exists between excessive cigarette smoke exposure and necrotic endothelial cells. With this taken into account, although no cytotoxicity was observed at the concentrations tested, changes on ultrastructural level are present and should be further investigated.

Keywords: Cadmium, Chromium, Lead, Heavy Metals, Scanning electron microscopy, Endothelial cells, MTT Assay, Crystal Violet Assay, DCFH-DA Assay, EA.hy926

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List of Abbreviations and Symbols

%	Percentage
µg	Microgram
µM	Micromole
AAPH	2'-azobis(2-amidinopropane) dihydrochloride
ADME	Absorption, distribution, metabolism and excretion
Al	Aluminium
ARDS	Acute respiratory disease syndrome
As	Arsenic
BCA	Bicinchoninic acid
BM	Basement membrane
Cd	Cadmium
Cr	Chromium
Cr (III)	Trivalent chromium
Cr (VI)	Hexavalent chromium
Cu	Copper
CV	Crystal Violet
DCF	2',7'-dichlorofluorescein
DCFH-DA	2',7'-dichlorodihydrofluorescein diacetate
ddH_20	Double distilled water
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxy-ribonucleic acid
EA	Endothelial cells
EA.hy926	Human umbilical vein endothelial cell line
GSH	Glutathione
H_2O_2	Hydrogen peroxide
H9-EC's	H9 human pluripotent stem cell-derived endothelial cells
Hg	Mercury
HUVEC	Human umbilical vein endothelial cells
IARC	International Agency for Research on Cancer

L	Litre
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
Min	Minutes
Mn	Manganese
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NED	*N*-1-napthylethylenediamine dihydrochloride
NO	Nitric oxide
$O_2^{\bullet-}$	Superoxide
$OH^{\bullet-}$	Hydroxyl radical
Pb	Lead
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
PI	Propidium iodide
PS	Phosphatidylserine
ROS	Reactive oxygen species
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy
V	Vanadium
WHO	World Health Organisation
Zn	Zinc

Chapter 1: Introduction

Metals are present in trace amounts in living tissues and are known to be naturally occurring inorganic elements, but are important in many vital processes of life (1). Metals also form part of many proteins and act as co-factors to enzymes and play a role in the water balance in the body (1).

Toxic metals, to a large extent, are dispersed in the environment through industrial and organic wastes, refuse burning, the use of transport modalities and power generation (2). Metals can travel far away from its source, either through mining debris or contamination by wind, depending on whether they are in a gaseous form or solid state. Metallic pollutants can therefore be washed out of the air into soil or find its way into various water ways (2).

Another source of heavy metal exposure is cigarette smoke. Tobacco smoke has carcinogenic, toxic and genotoxic properties. Cigarette smoke is known to contain both inorganic and organic human carcinogenic compounds. Comprising of about 4000 identified chemical compounds, cigarette smoke is very dangerous and toxic to human health (3). Several heavy metals have been identified in tobacco smoke including cadmium (Cd), chromium (Cr), lead (Pb), copper (Cu), mercury (Hg), manganese (Mn), aluminium (Al), zinc (Zn) and nickel (Ni) (3-5). Of these metals, the three most abundant metals are Cr, Cd and Pb (3, 6, 7).

This study was focused on these three metals on their own as well as part of metal mixtures because of their abundance in cigarette smoke. Each of these metal combinations were tested on the EA.hy926 cell line using five different techniques.

Even though the prevalence of cigarette smoking has somewhat decreased in several countries over the last decade, it still remains a serious hazard to public health worldwide (8). The World Health Organization (WHO) has published estimates, stating that by 2050 there will be approximately one and a half billion smokers worldwide. The hazardous impact that cigarette smoke poses on human health is well known, and is related to a wide range of disorders and diseases throughout every organ system in the human body (8). A survey conducted in 2017, found that an estimated 20% of South African adults, aged above 16 years of age are chronic cigarette smokers (9). Another South African study built on this statistic, where they investigated non-smokers exposure to second hand smoking. The results indicated that about 47% of non-smokers has reported exposure to SHS in at least one location (10).

Previous studies indicated that in a population groups living in close proximity to a mine, or individuals in careers such as smelting, had an increased level of Cd, Pb and arsenic in their

blood and urine samples (11). This indicates the potential harm, as well as accumulation of heavy metals through smoking, that can have an effect on the environment and contribute to the public health burden.

Although many studies have been done to test the effect of single metals on various organ systems such as the urinary system, clotting factors in blood and the effect on the morphology of different organ systems (12-18), little research has been done on the effects of these metals in combination. Previous studies investigated the effect of Cd and Cr on liver tissue where the results indicated hepatotoxicity shown by sinusoid dilations and tissue necrosis (18). The effects of Cd and Hg exposure on the coagulation system in Sprague-Dawley rats was also investigated. Results showed that the exposure to these metals affected fibrin fibre network formation which could contribute to the development of cardiovascular disease (16). In another *ex vivo* study, the oxidative and haemostatic effects of exposure to Cu, Hg and Mn, was investigated. This study determined the effects of the metals acting as catalysts of the Fenton reaction and/or the capability to bind to glutathione (GSH). It was concluded that Cu alone and in combination with Mn and Hg induced hydroxyl radical formation and binds GSH, representative of the capability of both these metals to illicit oxidative damage (12).

In this study, the focus was on the effect of Cd, Cr and Pb alone and in combination on endothelial cells. Endothelial cells are a single layer of flattened, highly specialized epithelial cells that line all blood vessels (19). The endothelium separates the circulating blood from exposure to sub-endothelial pro-thrombotic extracellular matrix components. Endothelium also secretes or expresses factors that modulate platelet reactivity, coagulation, fibrinolysis, and vascular contractility, all of which contribute to thrombotic formation. Such factors include nitric oxide, prostacyclin, von Willebrand factor, thrombomodulin and endothelin (20).

Damage to endothelial cells releases tissue factor which initiates blood clotting through the activation of von Willebrand factor, leading to platelet activation thrombus formation. Von Willebrand factor is a large, multimeric glycoprotein and is known to facilitate the adhesion of platelets to laminin and collagen in the sub-endothelial tissue and endothelin, a potent vasoconstrictor, following blood vessel damage (21, 22).

In response to tobacco smoke exposure, endothelial cells release inflammatory and pro-atherogenic cytokines linked to endothelial dysfunction (23). Endothelial dysfunction can be defined as a type of non-obstructive coronary artery disease where there are no artery blockages close to the heart, however, the larger blood vessels located on the heart's surface constrict rather than dilate. This dysfunction however, is not only limited to coronary circulation but can be seen

in any other vascular beds throughout the body (24). Endothelial dysfunction caused by heavy metal toxicity is directly related to the production of reactive oxygen species (ROS) leading to endothelial cell loss through necrosis or apoptosis (23).

The literature review will focus on the toxicity of heavy metals as well as the absorption, distribution, metabolism and excretion (ADME) of the metals. The known effects on human health and the metal composition of cigarettes will also be elucidated. Furthermore, the formation of ROS and the role of nitric oxide in apoptotic and necrotic pathways will be explained as well as the functioning of endothelial cells and the consequences of heavy metal exposure.

Chapter 2: Literature Review

2.1. Heavy metal characteristics and applications

The term heavy metal can be defined as “any metallic chemical element that has both a relatively high density and is poisonous or toxic at low concentrations” (5). Heavy metal elements are inherent components of the environment as they form part of the earth’s crust. Removal of heavy metals from the environment is problematic and is further worsened by the addition of more heavy metals through anthropogenic activities (2).

The effect that these toxic agents have on human health is at present an area of interest due to their abundance in the environment. This is because of the increase in the use of a wide assortment of metals in industry and processes in our daily life (2). Heavy metals are considered dangerous to human health as they have the ability to bio accumulate in tissues, causing toxicity (5).

Humans come in direct contact with heavy metals either by ingesting contaminated food, drinking contaminated water, through occupational exposure at the workplace or inhaling polluted air as dust fumes (25). The chain of contamination of heavy metals naturally follows a cyclic order; starting from industry, which is exposed directly to the atmosphere, water, food and soil and ultimately ending with human uptake (25). People living in close proximity to mines or mining areas are exposed on a daily basis to different heavy metals by using contaminated water for drinking, bathing, growing crops etc. (5). Also, people are often exposed to different metals at the same time, and at different concentrations.

2.1.1 Cadmium

Cadmium melts and boils at relatively low temperatures; its vapour is deep yellow and monatomic. This metal is typically used in the manufacturing of alloys, coatings, batteries, pigments and the production of plastics. The majority of Cd is used as the electrode component in the manufacturing of alkaline batteries (25, 26).

Cadmium is released into the environment via industrial practises and from Cd-smelters into fertilizers, groundwater and sewage sludge which has the ability to stay in both soils and sediments for many decades, where the Cd will eventually be taken up by plants. Consequently, substantial human exposure to Cd can be linked to the consumption of contaminated foods,

specifically fruits, cereals, leafy vegetables, grains and several contaminated beverages. Similarly, humans may be exposed to Cd through the inhalation of the deposits of burning of municipal waste or through the smoking of tobacco products (25).

2.1.2 Lead

Lead is a bluish, bright silver metal when placed into a dry atmosphere. The key contributors to Pb exposure include the smoking of cigarettes, contaminated food, industrial processes, drinking water and domestic sources. The industrial sources of lead include plumbing pipes, gasoline, storage batteries, lead bullets, pewter pitchers, toys and taps (25).

Lead is released into the atmosphere from both industrial processes and vehicle exhausts. Consequently, causing the soil to take up these deposits where it can flow into water bodies leading to human consumption (25).

2.1.3 Chromium

Chromium is a somewhat abundant element in the earth's crust; the free metal is never found in an unbound form in nature (27). Chromium is present in chromium steel, petroleum, coal, pigment oxidants, and deposited into the environment by oil well drilling, fertilizers and metal plating tanneries. Chromium is mostly used in manufacturing trades such as the production of paints and pigments, wood preservation, metallurgy, electroplating, tanning, pulp- and paper production and chemical production. These trades contribute to the large percentage of Cr pollution which has an adverse effect on ecological as well as biological species (25). The use of fertilizers and the incorrect disposal of sewage could contribute to the release of Cr into the environment (25). As a result, these agricultural and industrial practices increase both the environmental and agricultural contamination of Cr (26).

2.2. Absorption, Distribution, Metabolism and Excretion of heavy metals

Once heavy metals are ingested, they are immediately oxidized by the acidic medium of the stomach. The various oxidative states can freely bind to any biological particles and molecules such as enzymes and proteins to form strong and stable bonds (25). The most common functional group that heavy metals tend to bind to is the thio groups (SCH3-group of methionine and the SH-group of cysteine) (25).

2.2.1 Cadmium

Once in the circulatory system, Cd binds to albumin and blood cells, and then to metallothionein in both liver and kidney tissue (25). Cadmium has the potential to inhibit human thiol transferases like glutathione reductase, thioredoxin reductase and thioredoxin *in vitro* by binding to the cysteine residues in their respective active sites (25).

Exposure to cadmium also occurs through the smoking of tobacco products. Tobacco smoke moves cadmium into the lungs with inhaled air, where it will then be transported throughout the rest of the body. As this transport takes place via the bloodstream, it can increase the effects by potentiating Cd that is currently present in the system from Cd-rich food such as potatoes, chocolate and cereals (26).

2.2.2 Lead

Lead and its compounds are toxic and are retained by the body, accumulating over a long period of time, a phenomenon known as cumulative poisoning, until a lethal quantity is reached. The toxicity of Pb compounds increases as their solubility increases (26). Lead has no vital function in the human body, it can only have a toxic effect after uptake from food, water or air (26). Via the bloodstream, lead is dispersed between three main compartments, namely; soft tissue (that includes the brain, liver, bone marrow, and kidney), the blood and mineralized tissue such as bone and teeth. Thereafter, Pb is absorbed into blood plasma where it enters the blood cells. About 99% of the Pb concentration in blood are present in erythrocytes and 90% of the total body load of Pb is found in the skeleton (26).

2.2.3 Chromium

Chromium, specifically, trivalent chromium Cr (III), is an essential trace element which has been linked to improving sugar metabolism through the stimulation of insulin, increases in enzyme activity and serves a vital function in carbohydrate metabolism through stimulation of cholesterol and fatty acid synthesis (24, 26).

Chromium can be found in a variety of foods, including beef and poultry, milk and dairy - and whole grain products (26). Hexavalent chromium is a danger to human health, primarily for people who work in the textile and steel industry, as exposure can be hazardous. People who smoke tobacco also have a higher chance of exposure to Cr, as it is one of the main metals that are absorbed by and accumulates in various tissues, especially in the lungs (25).

Chromium is absorbed from the gastrointestinal tract by passive diffusion, transported from the bloodstream to tissues by the iron transport protein, transferrin, and transported from the tissues

back to the bloodstream and ultimately to the urine for elimination by the peptide low-molecular-weight chromium-binding substance (24).

2.3. Effects of heavy metals on health

Heavy metal toxicity has various health effects in the body by damaging and altering the functioning of various organs for example the kidney, brain, liver, lungs, and blood vessels (6, 26). Long-term exposure to heavy metals in the body can with time lead to severe physical, muscular and neurological degenerative progressions that are associated with diseases such as muscular dystrophy, Parkinson's disease, multiple sclerosis and Alzheimer's disease (25).

Heavy metal-induced carcinogenicity and toxicity comprises of many systemic aspects, most however, are not fully explained or understood. Each metal, however, is known to have distinctive physical and chemical properties that determine the mechanisms of action when the metal is involved in chemical processes (28). The following section will provide more information on the effect of Cd, Pb and Cr on human health.

2.3.1 Cadmium

Cadmium is a heavy metal that is usually detected in the environment in the water, soil and air. Increased Cd levels in water are absorbed by plants, ingested by animals and ultimately affect humans through the ingestion of both plant and animal materials (1, 26). Cadmium is associated with endocrine processes, which could affect the functioning of reproductive organs, including the placenta, testes and ovaries (29).Recurrent exposure to Cd leads to excessive accumulation in the kidneys, which may result in nephropathy and renal damage (1). Cadmium has a biological half-life in the kidneys between ten and 30 years (29).

When smoking, Cd is converted to cadmium oxide, which is inhaled. Almost 10% of the Cd is then deposited in the lungs, and 20 to 50% is transported into circulation (5). Furthermore, high levels of Cd decreases calcium absorption that decreases bone density and can lead to severe kidney disruptions (1).

The human body is unable to excrete Cd, worsening the health effects of Cd exposure (30). Short-term exposure to Cd inhalation could lead to severe damage to the lungs and cause respiratory irritation. The ingestion of Cd in higher doses could lead to stomach irritation resulting in diarrhoea and vomiting. Long-term exposure to Cd causes Cd-accumulation in the lungs and skeleton of the body, subsequently causing lung-and bone damage (25, 26).

Cadmium-induced toxicity has been widely studied and Cd can induce apoptosis in various cell types. Growing evidence suggests that elevated serum levels of Cd correlate with risk of vascular diseases and endothelial cell dysfunction (30, 31).

2.3.2 Lead

Lead exhibits extreme toxicity even at very low exposure levels (26, 31). Smoking however is not the primary source of Pb uptake in humans, but plays an increasingly important role in toxicity (26). This is due to the reduction of Pb emissions originating from petrol with the introduction of unleaded petrol. Around 50% of total Pb uptake by humans are from petrol fumes, however, the consumption of Pb from food such as root vegetables has also been reported to contribute to the total Pb load (5).

Lead toxicity affects learning and memory via three processes. Lead can impair the memory and learning ability in the brain through the inhibition of the N-methyl-d-aspartate receptor and can block neurotransmission by inhibiting neurotransmitter release, blockage of the neuronal voltage-gated Ca^{2+} channels and decrease the abundance of the brain-derived neurotrophic factor (25, 26).

2.3.3 Chromium

Chromium is a trace element that the body needs in small amounts and can occur in numerous oxidation states, however, only the trivalent, Cr (III), and the hexavalent, Cr (VI), forms are the most commonly found in the environment (5, 24).

Hexavalent chromium, according to the International Agency for Research on Cancer (IARC), is a group 1 carcinogen. Consisting of mostly hydroxyl radicals, Cr (VI) can cause deoxyribose nucleic acid (DNA)-damage, though inducing single strand breaks and have possible cell transforming effects (5, 24). Sources of Cr include nuts, wholegrain cereals, shellfish, eggs, and fruits and vegetables are sources of chromium (2, 24).

Exposure to increased amounts of Cr compounds could lead to the development of nasal septal ulcers which commonly present in chromate workers (24). Exposure to Cr can also lead to the inhibition of erythrocyte glutathione reductase, which then inhibits the ability to reduce methaemoglobin to haemoglobin (24). Both *in vivo* and *in vitro* tests have indicated that Cr compounds cause DNA damage which in turn leads to the formation of chromosomal aberrations, DNA adducts, changes in transcription of DNA and replication sister chromatid exchanges (23).

2.4. Heavy metals present in tobacco smoke

Cigarettes are made up of paper, tobacco and several additives. Approximately, 600 to 1400 additives are used in cigarette manufacturing alone, with a lot of these additives comprising of environmental pollutants such as Cd, Hg and Pb (4). A variety of toxic metals are found in tobacco which is dependent on the soil content of the tobacco plant. Fertilizer use are to be blamed for the high concentrations of Cd, Pb, Hg, As, Ni and Se in tobacco. The tobacco plant accumulates and absorbs heavy metals directly from the soil into its leaves (4). Tobacco smoking is the most evident source of Cd exposure in the general population (3). Cigarette smoke comprises of gases and particles produced by the burning of its numerous components at high temperatures (4).

A study conducted by Ashraf (3) in 2012, assessed the levels of selected heavy metals in popular cigarette brands sold and/or produced in Saudi Arabia through the use of graphite furnace-atomic absorption spectrometry. Their results indicated that a common constant was established between Cd and Pb being of the highest concentration of heavy metals in all cigarette brands tested (3).

Yaprak *et al* (6)., determined whether platelet-rich fibrin contained heavy metals and whether smoking increases heavy metal concentrations. The results indicated that smokers had significantly higher Pb, Cd, Cr, Mg, arsenic (As) and Vanadium (V) levels than non-smokers (6).

2.5. Endothelial Cells

Endothelial cells are classified as a single cell layer that lines all blood vessels and regulates exchanges between the bloodstream and the surrounding tissues (32). The endothelium is considered to be both one of the largest and most heterogeneous organs in the human body. Endothelium functions in the delivery of nutrients and oxygen to all the tissues in the body (33).

Endothelial cells maintain an anti-thrombogenic (inhibitory to clot formation) and selectively permeable barrier where they regulate where and when white blood cells leave the circulation and travel to the interstitial space of tissues. They also secrete a large selection of paracrine factors for growth of neighbouring cells, vessel constriction and dilation linked to the functioning of the tunica media (21). Vascular endothelial cells are squamous, polygonal, and elongated with the long axis in the direction of blood flow (19).

New blood vessels mature from the walls of current small vessels through the outgrowth of endothelial cells. Endothelial cells have the capability to form hollow capillary tubes even when isolated in tissue culture. The endothelial cells of developing veins and arteries have the same

cell-surface proteins, which each control the way in which they link up to generate a capillary bed (32).

As mentioned, in response to smoke exposure, endothelial cells release inflammatory and pro-atherogenic cytokines, all of which leads to endothelial dysfunction (23). Endothelial dysfunction is a type of non-obstructive artery disease, which shows no artery blockages close to the heart, however, the larger blood vessels located on the heart's surface constrict rather than dilate. This is however not limited to coronary arteries alone, but can be seen in vascular beds throughout the body (23, 24, 34, 35).

Regenerated endothelium exhibits impaired endothelium-dependent relaxation and a greater tendency to display endothelium-dependent constrictions. These endothelial cells show a greater production of oxygen-derived free radicals (ROS), a reduction in nitric oxide release and modified low-density lipoprotein metabolism (36).

In this study, EA. Hy926 cells (human umbilical vein cells) were used. The EA. Hy926 cell line was first established by combining the primary human umbilical vein cells with a thioguanine-resistant clone of A549 by exposure to polyethylene glycol (PEG) (37). Endothelial cells contain Weibel-Palade bodies, which act as small storage granules located in endothelial cells comprising the intima of the heart and blood vessels. They are found in arteries, veins, capillaries and the endocardium, but notably not in the lymphatic vessels. These bodies function to store two principal molecules, P-selectin and von Willebrand factor (38).

Endothelial cells depend on endothelial nitric oxide synthase for its consistent activity and function, as well as for its homeostasis and vasculature integrity as a whole. Furthermore, the nitric oxide activity can either be lost, or uncoupling of the enzyme can occur due to oxidative stress (33).

2.6. **Reactive Oxygen Species**

Certain heavy metals are known to produce free radicals that can lead to oxidative stress and in turn cause cellular damage. The mechanism of free radical generation is specific to the heavy metal element (25). Oxidative stress is caused by the intracellular presence of ROS which overwhelms the natural anti-oxidant defence of the cell (39). Reactive oxygen species are mostly produced as a by-product of various cellular processes in multiple forms including superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\bullet -}$) (39). Metal-induced toxicity can create an imbalance of antioxidant mechanisms in living tissues which leads to the generation of

large quantities of ROS. This antioxidant imbalance lead to the breakdown of nucleic acids, proteins, and lipid peroxidation. Several human diseases such as diabetes, cancer, neuro-degenerative and auto-immune diseases have been linked to oxidative stress by ROS (1, 40). This is true for Cd as it mainly damages cells through the production of ROS, which leads to single-strand DNA damage and disrupts proteins and nucleic acid synthesis (28).

Chromium, mainly Cr^{4+} has been shown in *in vitro* studies to produce free radicals from H_2O_2 (25). Similarly, *in vivo* studies indicated an increase in free radicals due to the presence of Cr^{4+} alone in the blood and liver tissues of animals (25).

The exact mechanism of Pb-induced oxidative stress consists of an imbalance between the production and elimination of ROS in cellular components and tissues leading to the damage of DNA, proteins and membranes (31). Pb toxicity is associated with oxidative stress causing damage to the lipid membrane of cells. It has been hypothesised that Pb has the potential to elongate arachidonic acid that is present in the cell membrane. This change in arachidonic acid, can cause increased lipid peroxidation in the cells (31).

Endothelial cells are also predisposed to oxidative stress, which is induced by circulating leucocytes in the blood, such as neutrophils, that produce a large amount of ROS to kill invading pathogens (41). Literature suggests that a modification in endothelial cell function by oxidative stress increases the susceptibility to both cardiovascular and central nervous system diseases. This susceptibility to chronic diseases is amplified with the exposure to air pollution (41).

Endothelial-derived NO is a potent vasodilator as it is a vital controller of endothelial cell homeostasis. The loss of NO bio-availability caused by the decreased synthesis or increased degradation has been identified to be a vital factor for vascular endothelial dysfunction, which contributes to the pathological development of atherosclerotic cardiovascular diseases (35).

2.7. **Apoptosis & Necrosis**

Cell death can be divided into two main processes, namely, apoptosis which is also known as programmed cell death and necrosis which is known as uncontrolled or accidental cell death (42). Apoptosis is an energy-dependent process characterised by nuclear condensation and fragmentation, cell shrinkage and cellular fragmentation in the form of apoptotic bodies, which are later removed by phagocytic cells (42). The key difference between apoptotic and necrotic cell death is that necrotic cell death is characterised by plasma membrane leakage, leading to the

prevalence of inflammation in the affected area, due to the release of intracellular components (42).

Literature suggests that carcinogenic transition metals such as Cd and Cr are known to cause apoptosis along with DNA base modifications, rearrangements and strand breaks (43). It has also been hypothesised that apoptosis in Pb toxicity takes place via the inhibition of Bcl-2 and the activation of caspase-3 following mitochondrial cytochrome C release (44).

In this study, morphological analysis of apoptotic or necrotic cell death was determined using SEM, by viewing the morphological changes of the endothelial cells with exposure to varying metal concentrations. Cell death was further quantified using propidium iodide (PI) and Annexin V with flow cytometry.

2.8. **Previous studies**

A study conducted by Cao *et al.,* (45), tested the toxic effects of Cr(VI) on bovine haemoglobin and human vascular endothelial cells (HUVEC's). Various concentrations of Cr (VI) were used, namely, 10, 20, 40, 60, 80,100, 150 and 200 mM. The results indicated that Cr (VI) could induce oxidative stress in HUVECs, however, the viability of HUVECs decreased significantly in all the groups treated with Cr (VI) compared to the control group (45).

In 2020, Cao *et al*., (46), tested the inhibitory effects of taxifolin against Cr(VI)-induced cell damage in HUVECs by using the MTT, DCFH-DA, and cellular adhesion assays as well as Western Blot analysis (46). The authors concluded that taxifolin was a potential agent to prevent endothelial dysfunction, monocyte inflammation and cell adhesion induced by Cr(VI) (46).

Tang *et al.,* (30), examined the mechanisms of Cd-induced endothelial toxicity in the H9 human pluripotent stem cell-derived endothelial cells (H9-ECs) (30). To determine toxicity, the authors made use of the TUNEL assay, scratch assay, caspase 3 assay, cell viability assay, cell cycle and apoptosis assay, tube formation assay, cell proliferation assay, western blot analysis and RNA sequencing. The results indicated that H9-ECs were prone to cadmium chloride ($CdCl_2$) induction, resulting in harmful changes of cell structure and significantly elevated levels of apoptosis. Cadmium chloride treated H9-ECs gave rise to an endothelial cell dysfunction phenotype and significantly differential transcriptomic profile (30).

Zhong *et al* in 2017, (35) determined the metabolic profile of non-toxic Cd-induced HUVECs. HUVECs, consisting of six per group, were treated with 0, 1, 5, or 10 µM $CdCl_2$ for 48 h (35). To determine the effects of Cd, LDH activity, cell viability and intracellular total protein concentrations

were independently determined using the CCK-8 cell viability assay, enhanced bicinchoninic acid (BCA) protein assay and LDH colorimetric assay. Cell tube formation assay, nitric oxide colorimetric assay and Western Blot analysis was done to examine the full effect that Cd has on HUVECs. The results showed that when compared to control HUVECs, Cd-exposed HUVECs were dysfunctional, showing decreased NO production, non-significant oxidative stress and a pro-inflammatory state (35).

In 2017, Venter *et al*.,(13), conducted an *ex vivo* study to test the exposure to heavy metals Cd and Cr alone and to also identify whether Cr synergistically rises the effect of Cd on important physiological practices such as blood coagulation. The researchers reported ultrastructural changes in the fibrin networks, erythrocytes and platelets. Varied erythrocytes morphologies, thicker fibrin fibres and activated platelets were observed. It was concluded that blood does serve as an important target for Cr and Cd toxicity (13).

Another study in 2017 by Arbi *et al*., (16), tested the effects of Cd and Hg exposure on the coagulation system in Sprague-Dawley rats and linked this possible toxicity with the formation of cardiovascular disease (CVD). Upon SEM analysis, it was reported that both metals caused platelet activation. Further analysis concluded that Cd increased fibrin fibre thickness, causing the accumulation and formation of dense matted deposits. Hg reduced fibrin formation, whereas in the combination group Hg increased the effect of Cd. It was concluded that exposure to these metals, had effects on fibre network formation, contributing to the development of CVD (16).

In 2019, Van Rensburg *et al*., (12), conducted a study testing the oxidative and haemostatic effects of exposure to Cu, Hg and Mn, in an *ex-vivo* study. This study tested the effects of the metals acting as catalysts of the Fenton reaction and/or ability to bind glutathione (GSH). It was concluded that Cu alone as well as in combination with Mn and Hg causes hydroxyl radical formation and binds GSH, representative of the ability of both metals to induce oxidative damage (12).

A study conducted by Tubsakul *et al.,* (47), tested the effect of curcumin on oxidative stress, alteration of vascular responsiveness and hypertension induced by exposure to either Pb, Cd or the combination of Pb and Cd. Curcumin is a polyphenolic compound with strong antioxidant activity. Male Sprague-Dawley rats were exposed to low levels of Cd-chloride and/or lead acetate in the drinking water for a total of 16 weeks, whereas curcumin was intragastrically administered through injection into the stomach once daily for the last four weeks. The results showed that exposure to Cd or Pb or the combination caused an increase in blood pressure as well as peripheral vascular resistance, and a decreased blood pressure response was noted with the

intravenous infusion to acetylcholine. Interestingly, they found that curcumin decreased the metal levels in the aorta, blood, liver and kidney, determined by using inductively coupled plasma mass spectrometry. This was representative of the usefulness of curcumin as a dietary supplement for defence against the noxious effects of the heavy metals (47).

A study conducted by Fotakis and Timbrell compared the cytotoxicity of $CdCl_2$ (0-300 µM) using four different cytotoxicity assays (LDH, MTT, Neural Red and a Protein Assay) on two different cell lines, namely HTC and HepG2 cells (48). No cytotoxicity was seen for LDH leakage, the MTT and the protein assay, whereas for the neutral red assay cytotoxicity was seen after 3 h of incubation (48). With the HepG2 cells, MTT showed cytotoxicity after 3 h, whereas no cytotoxicity was seen for any of the other assays after 3 h. It was then concluded that based on the assays and experiments done that the MTT and neural red assays were the most sensitive in detecting cytotoxicity in cell culture (48).

Based on the research mentioned above, it is evident that extensive research has been done on Cd, Pb and Cr specifically at high concentrations, however limited research is available on the daily dosage of metal exposure via cigarette smoke and how this can affect the morphology and viability of cells, specifically endothelial cells.

2.9. **Aim**

The aim of this study was to investigate the effects of Cd, Pb and Cr, alone and in combination on the endothelial cell line, EA.hy926, by determining the cytotoxicity and, the ability of these metals to produce ROS. Furthermore, to investigate the ability of these metals to induce apoptosis and necrosis and the possible morphological alterations associated with these cell death processes.

2.10. **Objectives**

The aim of the study was achieved by the following objectives:

1. To investigate the effect of these metals alone and in combination on cytotoxicity of the EA.hy926 endothelial cell line by using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) and Crystal Violet (CV) assays.
2. To investigate the effect of these metals alone, and in combination on the production of reactive oxygen species (ROS) in the EA.hy926 endothelial cell line through the 2', 7'-dichlorodihydrofluorescein diacetate (DCFH-DA) assay.

3. To investigate the effect of these metals alone, and in combination on the induction of apoptosis and necrosis in the EA.hy926 cell line using Annexin V and the Propidium Iodide (PI) Assay.
4. To investigate the effect of these metals alone, and in combination on the morphology of the EA.hy926 endothelial cell line by using scanning electron microscopy (SEM).

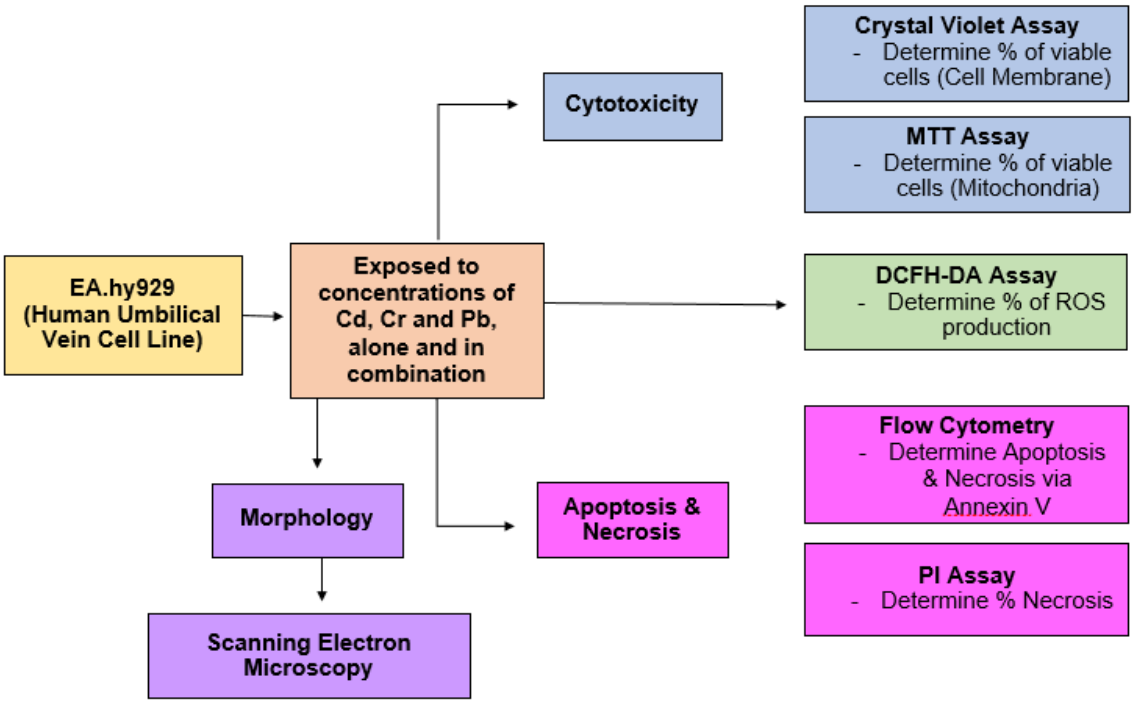


Figure 2.2.1- Flow diagram of the study

Chapter 3: Materials and Methods

3.1. Materials

All metals used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA), from which stock solutions were created and diluted using sterile ddH_2O. Gibco Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin and trypleEx were purchased from Gibco Life Technologies (Waltham, MA, USA). 3-(4, 5-di-methylthiazol-2-yl) 2, 5-diphenyltetrazolium bromide (MTT), 2, 2'-azobis (2-amidinopropane) dihydrochloride (AAPH), Crystal Violet (CV), dichlorofluorescein diacetate (DCFH-DA), Annexin V (FITC) and Propidium Iodide (PI) dyes were obtained from Sigma-Aldrich (Atlasville, SA).

Disposable plastic ware included: 96 well plates, 24 well plates, fluorescent 96 well plates, 25 cm^2 and 75 cm^2 tissue culture flasks, 50 ml and 15 ml centrifuge tubes, FACS tubes, 1.5 ml Eppendorf tubes, 50 ml, 15 ml tubes and pipette tips (10, 25, 100, 200, and 1000 µL) which were obtained from Greiner Bio-one (LASEC, Cape Town, SA).

Equipment used included: a FLUOstar OPTIMA plate reader from BMG lab technologies, (Offenburg, Germany), A Hermle Z300 centrifuge (LASEC, Cape Town, SA), A Zeiss Ultra Plus FEG SEM (Oberkochen, Germany), Flow Cytometer (Beckman coulter, Germany). Glassware was sterilized at 121°C for 20 min in a Prestige Medical Autoclave (series 2100).

3.2. Metal Preparation

The concentrations of Cd, Cr and Pb were obtained from the study conducted by Yaprak *et al* (6), as indicated in Table 3.1 below (in µg/L). These concentrations were used as the study investigated the circulating metal concentrations in the blood of chronic smokers. Stock solutions (concentrations were equal to X2 the pre-determined values for each respective heavy metal) for the various heavy metals were made using sterile ddH_2O. Working solutions were then made from these stock solutions. The endothelial cell line, EA.hy926 cell line, was then exposed to the different concentrations of heavy metals as indicated in Table 3.1.

Table 3.1- The initial concentrations of Cd, Cr and Pb that were exposed to the EA.hy926 cell line.

GROUP	CONCENTRATION (X0.1)	CONCENTRATION (X1)	CONCENTRATION (X2)
Control	0 µg/L	0 µg/L *	0 µg/L
Cd	0.021 µg/L (1.15 x 10^{-4}µM)	0.21 µg/L * (1.15 x 10^{-3} µM)	0.42 µg/L (2.29 x 10^{-3} µM)
Pb	0.14 µg/L (5.03 x 10^{-4} µM)	4.1 µg/L * (0.015 µM)	8.2 µg/L (0.029 µM)
Cr	22.4 µg/L (0.182 µM)	224 µg/L * (1.82 µM)	448 µg/L (3.65 µM)
Cd + Pb	0.021 µg/L + 0.14 µg/L	0.21 µg/L + 4.1 µg/L *	0.42 µg/L + 8.2 µg/L
Cd + Cr	0.021 µg/L + 22.4 µg/L	0.21 µg/L + 224 µg/L *	0.42 µg/L + 448 µg/L
Pb + Cr	0.14 µg/L + 22.4 µg/L	4.1 µg/L + 224 µg/L *	8.2 µg/L + 448 µg/L
Cd + Pb + Cr	0.021 µg/L + 0.14 µg/L + 22.4 µg/L	0.21 µg/L + 4.1 µg/L + 224 µg/L *	0.42 µg/L + 8.2 µg/L + 448 µg/L

*concentrations obtained from Yaprak *et al* (6).

3.3. Culture of Endothelial Cells

The EA.hy926 endothelial cell line was obtained from the Department of Physiology, Faculty of Health Sciences, University of Pretoria and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), (Sigma-Aldrich, St Louis, MO, USA) and incubated at 37°C in humidified atmosphere with 5% CO^2. The cells were then trypsinized and passaged once reaching 75 to 90% confluence. For measurements, cells suspended in medium was seeded onto plates at a density of 5×10^4 per well for all experiments (46, 49).

The Trypan Blue exclusion method was used to determine cell density. The endothelial cells were then exposed to the different concentrations of metals in triplicate. Control cells were exposed to ddH_2O containing no metals, and the assays respective positive controls.

3.4. MTT Assay

Cell viability can be defined as “a measure of the proportion of live, healthy cells within a population” (50). Cell viability assays are used to determine the overall health of cells, following the addition of a test subject. The MTT assay provides an indirect measurement of cell growth and cell death through measurement of the ability of mitochondria to convert the tetrazolium salt into a coloured formazan product that may be measured spectrophotometrically (51).

After reaching confluence, 90 µL of cells were plated in 96 well plates at a concentration of 5 x 10^4 cells/ well and left overnight to attach. Thereafter, the cells were exposed to 10 µL the respective samples indicated in table 1 and left for 72 h. After the 72 h incubation, MTT solution, (5 mg/ml), (Sigma-Aldrich, St Louis, MO, USA) was prepared in PBS, and 10 µL was added to each well. The 96-well plate was incubated at 37 °C, 5% CO_2 for 3 h. MTT was then removed

along with the medium and gently blotted dry to remove any excess water. Thereafter, 100 µL of 25% (v/v) DMSO prepared in ethanol was added to each well, and absorbance was measured using the FLUOstar OPTIMA plate reader (BMG Labtech, Offenburg, Germany) at 570 nm. The results of this assay were calculated as a percentage of the control and indicated in Figure 4.1.

3.5. Crystal Violet

Cells which are adherent tend to detach from the culture plates during cell death. Preserved adherence is evaluated using a crystal violet assay (51).

After reaching confluence, 90 µl of cells were plated in 96 well plates at a concentration of 5 x 10^4 cells/ well and left overnight to attach. Thereafter, the cells were exposed to 10 µl the respective samples indicated in table 3.1 and left for 72 h. After the 72 h incubation, 10 µL of 20% formaldehyde was added to the cells to complete fixation. The plate was then incubated for 30 minutes at 37 °C and 5% CO_2. The fixative was thereafter removed and blotted dry. Cells were then stained by placing 100 µL of 0.1% (w/v) crystal violet solution prepared in 200 mM (0.75%) formic acid and left at room temperature for 30 minutes. The dye was then removed from the cells, washed with running water, and blotted dry. The bound dye was dissolved in 100 µL of 10% acetic acid in ddH_2O. The absorbency was read at 630 nm using the FLUOstar OPTIMA plate reader (BMG Labtech, Offenburg, Germany). The results were calculated as a percentage of the control and is indicated in Figure 4.2.

3.6. DCFH-DA Assay

DCFH-DA is a cell-permeable dye that is non-fluorescent and is hydrolysed intracellularly into its polar, but non-fluorescent form DCFH on the action of cellular esterase and thus is retained in the cell. The oxidation of DCFH by the action of intracellular ROS and other peroxides turns the molecule into its highly fluorescent form 2',7'-dichlorofluorescein (DCF) that can be detected by various fluorescent methods (52).

For the DCFH-DA Assay, 100 µL of cells were plated at a density of 5.5 x 10^4 cells / well in a fluorescence 96 well plate (Sigma-Aldrich, St Louis, MO, USA) and left overnight to attach. A 75 µM DCFH-DA solution (Sigma-Aldrich, St Louis, MO, USA) was prepared beforehand and 50 µL were added to each well and incubated for 60 min. The extracellular DCFH-DA was then removed by washing the EA.hy926 cells in isoPBS (isoPBS: 0.137 M NaCl, 3 mM KCl, 1.9 mM NaH_2PO4, 8.1 mM Na_2HPO4, pH 7.4) (Sigma-Aldrich, St Louis, MO, USA). The washed cells were then exposed to 10 µL of each respective sample as indicated in Table 1, along with two controls, one

containing only ddH_2O, which served as the negative control and the other being AAPH (0.04g/ml), which acted as the positive control (Sigma-Aldrich, St Louis, MO, USA). Three replicates of each were done for 24 h, 48 h and 72 h exposure times. An endpoint reading was done after each respective exposure time by the FLUOstar OPTIMA plate reader (BMG Labotech, Offenburg, Germany) with an excited wavelength of 485 nm and emission wavelength of 520 nm.

A nitrate standard curve was prepared by diluting 0.1M $NaNO_2$ made up in ddH_2O. The net area under the curve for each sample was calculated using the AAPH alone as having caused 100% damage. The results for the DCFH-DA assays at each relevant time point reading is indicated in Figures 4.3 – 4.5.

After completion of the DCFH-DA assay at each respective incubation reading, a CV assay was done on the same plate to determine if any cell death has taken place.

3.7. **Annexin V Assay**

The Annexin V assay works on the principle of assessing loss of plasma-membrane asymmetry. Phosphatidylserine (PS) usually found in the inside of the plasma membrane is flipped to the surface of the cell during apoptosis. Annexin V has a strong Ca^{2+} affinity for PS and can therefore be utilised as a probe that binds to the flipped PS. Binding ability of Annexin V is assessed with flow cytometry. The protocol used for Annexin V was adapted from Ginting *et al*., 2021 (53), Liu *et al*., 2020 (54) and Karri *et al*., 2018 (55) as it was specialised for the EA.hy926 cell line. Due to time and financial restraints, only the X2 metal concentrations were analysed under the Annexin V assay.

Cells were plated at a concentration of 7 x 10^4 cells/ml, 9 ml of cell suspension was added to a 75cm^3 flask and left to attach overnight. Following overnight attachment, 1 ml of sample (dissolved in medium) was added to each flask to make up a final volume of 10ml. The sample was left to incubate for 72 h. Following the 72 h incubation period, cells were trypsinized and centrifuged at 12298 g-force (1000 rpm) for 5 minutes. The cell pellet was washed in ice-cold PBS (0.1 M) and centrifuged again at 12298 g-force (1000 rpm) for 5 minutes. Cells were then counted in ice-cold PBS and adjusted with binding buffer to make up 1 x 10^6 cells/ml. The cell samples were then analysed. For analyses, 195 µL of each cell pellet was placed in an Eppendorf tube along with 2.5 µL of both Annexin V and Propidium Iodide (PI) dye and then incubated in the dark for 5 minutes at 4 °C. The cell pellet was then centrifuged and washed with 200 µL of the binding buffer. Cells were then transferred to a FACS tube, vortexed and analysed using a Flow Cytometer (Beckman Coulter, Germany) with the fluorescence of Annexin V (FITC) and PI was measured

by logarithmic amplification. The number of events analysed for each gate/sample was 1500. Each experiment was conducted in triplicate, and data was analysed using Kalusa C Software (Beckman Coulter), the analysis is shown in Figure 4.7.

3.8. Propidium Iodide (PI) Assay

To substantiate the results obtained from the Annexin V assay with flow cytometry, an alternative method was used in-order to test for necrosis, using the propidium iodide (PI) dye and reading it as a fluorescence assay. Propidium Iodide is a red fluorescent dye that binds to DNA fragments of cells which already have a compromised cell membrane (56). The protocol used was obtained from the John Hopkins Medical Information website (56).

Cells were plated at a concentration of 5 x 10^4 cells/ml, with 90 µL of cell suspension added to each well in a 96 well fluorescent plate and left to attach overnight. Following overnight attachment, cells were exposed to 10 µl of sample and incubated for 72 h. After the 72 h incubation time, all contents in each well were removed carefully using a pipette tip and 200 µL of 2.5 µM of PI dye, diluted in warm (37 °C) PBS was added to each well. The fluorescent plate was then placed in the spectrophotometer at 37 °C and read for 1 h at 530 nm/ 620 nm. Results were analysed as percentage necrosis and indicated in Figure 4.8.

3.9. Scanning Electron Microscopy

Scanning electron microscopy (SEM) is a significant electron microscopy technique that is capable of achieving a very detailed visual image of the surface of a specimen with high-quality and spatial resolution. The sample is exposed in SEM to a high-energy electron beam which gives information about the morphology, composition, topography, etc. of a material (57, 58).

The effect of the metals, alone and in combination, on the morphology of EA.hy926 cells was investigated using SEM. Cells were grown on glass cover slips in 24 well plates at a concentration of 5 x 10^4 cells/ well, which have been coated with poly-L-lysine and fixed in 2.5% GA/FA in 0.075 M $NaPO_4$ buffer for 30 minutes. Cells were exposed to the relevant concentrations of metals indicated in Table 3.1 and left overnight. The cells were thereafter washed 3 times with 0.075M $NaPO_4$ buffer, for 10 minutes each. Osmium tetroxide (1%) (Sigma-Aldrich, St Louis, MO, USA), was added for 45 minutes, and washed thereafter 3 times with buffer, 10 minutes each. The cells were then dehydrated in increasing concentrations of ethanol (EtOH, Merck, Darmstadt, Germany), starting with 30% then 50%, 70%, 90% and 100% (three times), ten minutes each. Thereafter, 100% hexamethyldisilazane (HMDS) (Merck, Darmstadt, Germany) was added for 1

h, thereafter, one to two drops of HDMS were added on the glass cover slips and allowed to dry overnight. Samples were then mounted on aluminium stubs with carbon tape and coated with carbon three times. A Zeiss Ultra Plus FEG SEM (Oberkochen, Germany) was used to study the morphology of the samples.

3.10. Statistical considerations

This study was set out to investigate the effects of Cd, Pb and Cr, alone and in combination, each at three concentrations (X0.1, X1, and X2), on the EA.hy926 cell line by determining the cytotoxicity as well as the ability of these metals to produce ROS, necrosis and apoptosis. Morphological changes were assessed with scanning electron microscopy.

3.10.1. Sample size

The experiment was conducted by using a two-factor study design. The two factors are the addition of heavy metals at seven levels (Cd, Pb and Cr alone or in combination) and concentration at three levels (0.1X, 1X, 2X) to the EA.hy926 cell line, i.e. a 7x3 study design. There were at least three repeats, where in each repeat there are three replicates, and hence at least 7x3x3 = 63 observations. The appropriate analysis of variance (ANOVA) has at least 43 residual degrees of freedom, which is adequate in view of the norm that requires at least 30 degrees of freedom. Testing was done at the 0.05 level of significance.

3.10.2. Statistical Analyses

Data summary reports means, standard deviations, range and 95% confidence intervals by heavy metal level (Cd, Pb, Cr, Cd+Pb, Cd+Cr, Pb+Cr, Cd+Pb+Cr) and by heavy metal-concentration for the observed parameters. Data analysis, by parameter of interest, employed a two-way ANOVA with main effects heavy metal (seven levels) and concentration (three levels).

All statistical analyses were carried out by using Graph Pad Prism analysing software, version 8.4.3. After each experiment, a normality and logarithmic test was conducted using the normal (Gaussian) distribution, Anderson-Darling test and the D'Agostino- Pearson omnibus normality tests to determine the normality of the data with a significance level (alpha) set to 0.05. Once normality was determined, outliers were identified using the ROUT method with a Q-interval of 1% and all outliers were then excluded from the data set. The One-Way ANOVA and Tukey's multiple comparison tests were performed on all data sets. The Brown-Forsythe test and Bartlett's test were performed on all non-parametric data. Multiple comparisons were done between the

mean of each metal group and the vehicle control (ddH_2O), as well as the mean of each metal group independent of the control and between each metal group's different concentrations to determine significance. Statistical analysis was performed with a 95% confidence interval and a p-value of < 0.05 was considered significant.

Chapter 4: Results

4.1. Heavy Metals have no effect on cell viability

The cytotoxic effect of each metal concentration was determined on the EA.hy926 cell line exposed to the varying concentrations of each metal and incubated for 72h using the MTT assay. No significant difference was observed between any of the metal exposures and the untreated control (Figure 4.1).

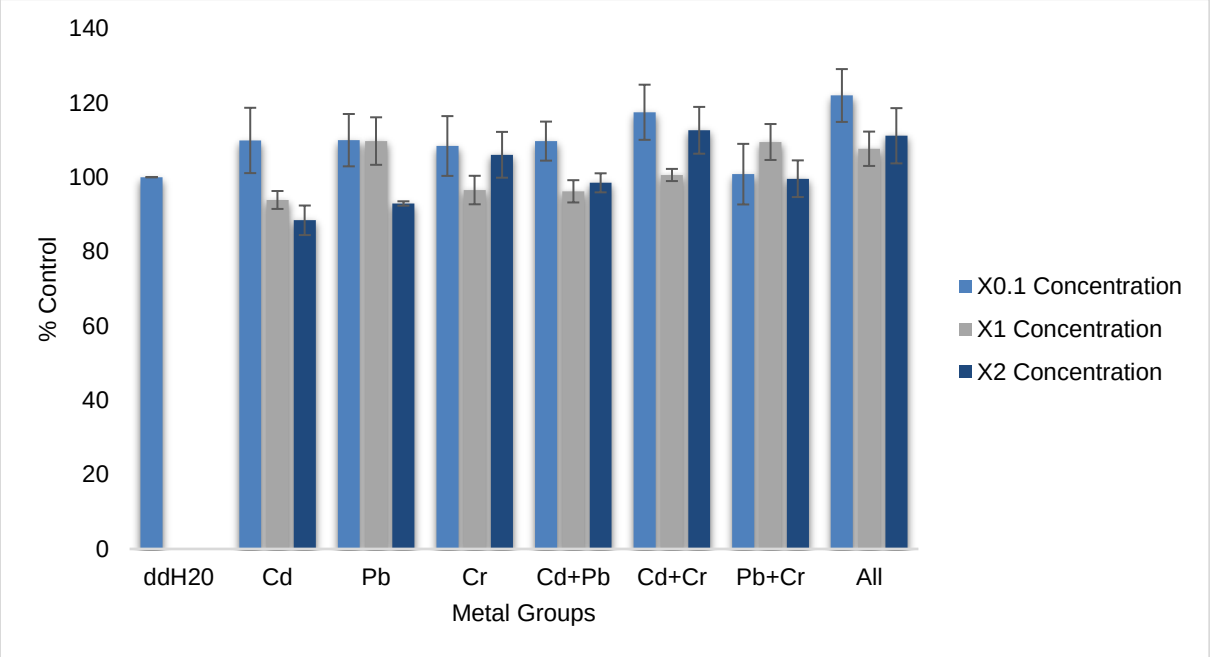


Figure 4.1- Graph indicating the cytotoxicity of each respective metal and each concentration using the MTT Assay with ddH_2O as vehicle control and expressed as a % of the control. Results are expressed as mean ± SE of three independent triplicate experiments with p-value of < 0.05.

The cytotoxic effect of each heavy metal group was further determined using the CV assay. The EA.hy926 cell line was exposed to the varying concentrations of each metal and incubated for 72h. No significant difference was observed between any of the metal exposures and the untreated control (Figure 4.2).

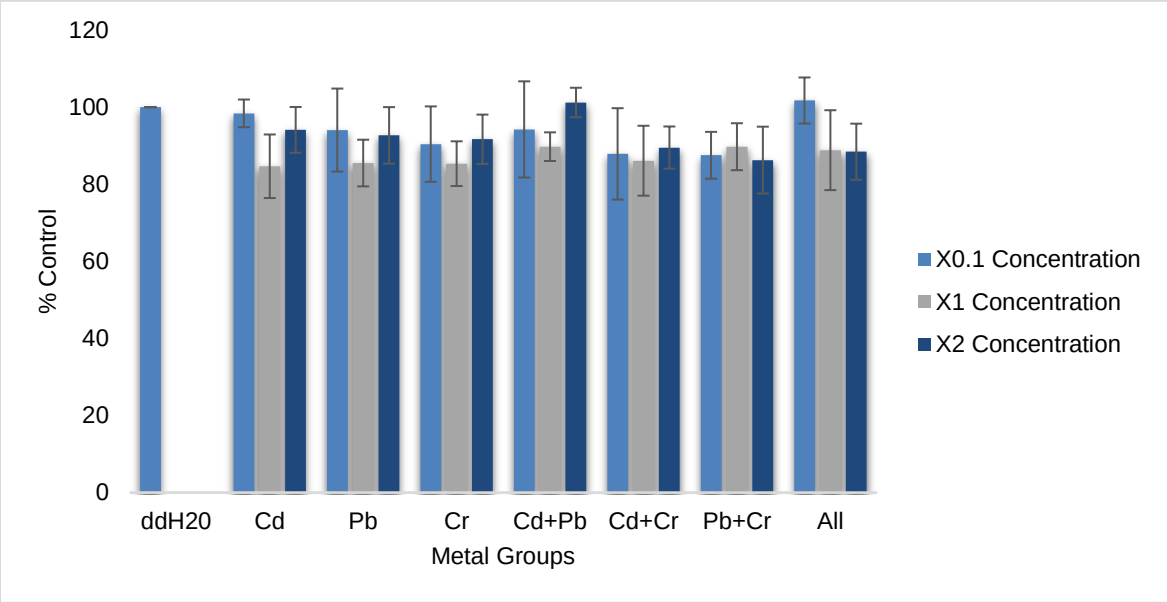


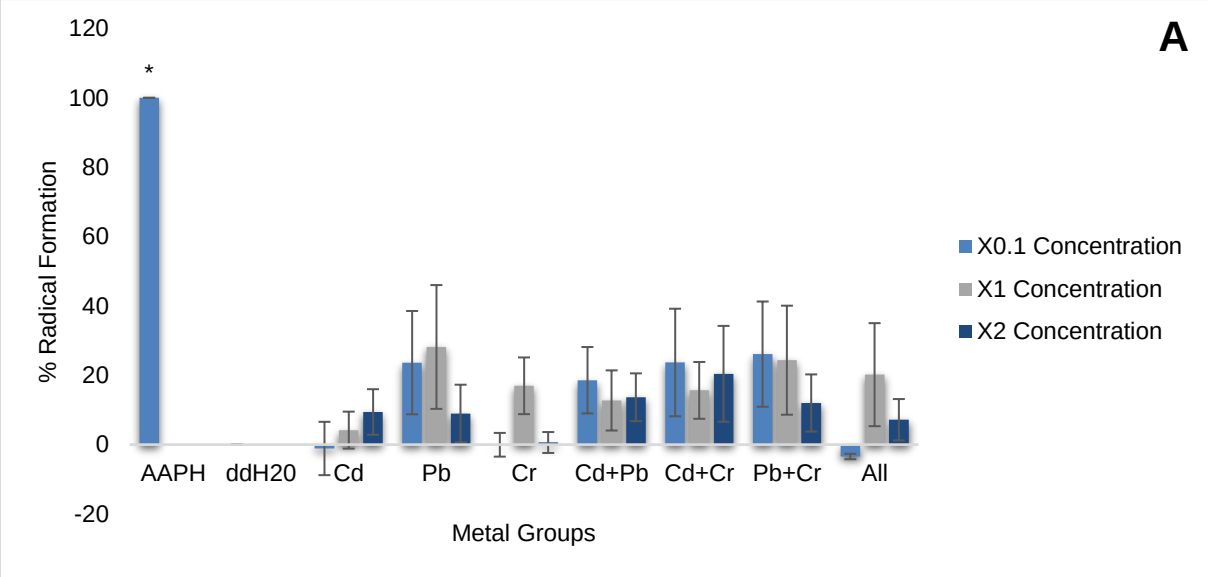
Figure 4.2- Graph indicating the cytotoxicity of each of the respective metals using the Crystal Violet Assay with ddH_2O as vehicle control and expressed as a % of the control. Results are expressed as mean ± SE of three independent triplicate experiments with p-value of < 0.05.

4.2. Heavy metals caused a limited increase in ROS production

The DCFH-DA assay was used to determine the % radical formation formed after exposure to the metals at 3 different time periods: 24 h, 48 h and 72 h.

4.2.1. 24 h exposure

The intracellular ROS content was assessed using the DCFH-DA assay. The standard DCFH-DA assay protocol was used. After a 24 h incubation period, the results were read at 520 nm and expressed as % radical formation (Figure 4.3 A). The corresponding crystal violet assay done after completion of the DCFH-DA assay was read at 630 nm and expressed as a % of the control (Figure 4.3 B). In both instances, ddH_2O was used as the vehicle control and AAPH as the positive control. No significant difference was noted between the vehicle control and any of the samples.



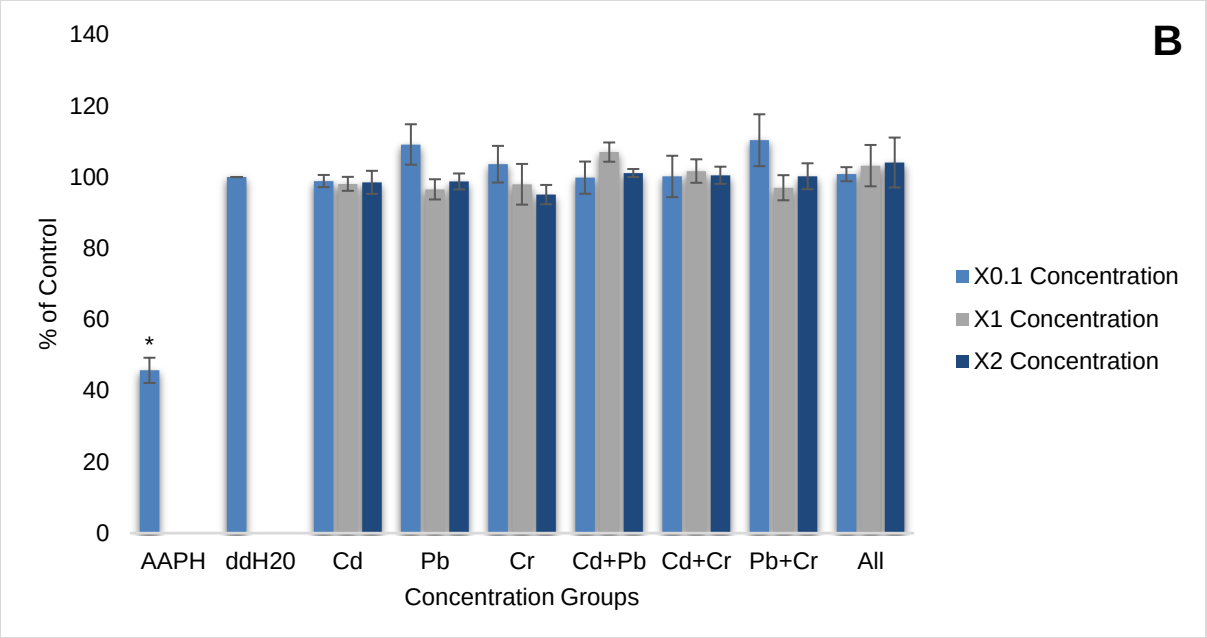


Figure 4.3 (A)- Graph indicating the % radical formation that each metal and their respective concentrations created using the DCFH-DA assay after 24h incubation with ddH_2O as the vehicle control and AAPH as the positive control. Results are expressed as mean ± SE of three independent triplicate experiments with p-value of < 0.05. * - Significance was seen between this group and every sample. **(B)-** Graph indicating the CV assay results based on the DCFH-DA assay results for 24h incubation, with ddH_2O as the vehicle control and AAPH as the positive control. Results are expressed as mean ± SE of three independent triplicate experiments. * - Significance was seen between this group and every sample.

4.2.2. 48 h exposure

The intracellular ROS content was assessed using the DCFH-DA assay. The standard DCFH-DA assay protocol was used. After a 48 h incubation, the results were read at 520 nm and expressed as % radical formation (Figure 4.4A). The corresponding CV assay done after completion of the DCFH-DA assay was read at 630 nm and expressed as a % of the control (Figure 4.4 B). In both in-stances, ddH_2O was used as the vehicle control and AAPH as the positive control. A distinctive pattern is seen in the Cr group as well as in the triple combination group, with an increase in % radical formation with the increase in metal concentration. A similar pattern can be seen in the Pb group, but is not as distincitive. Significance is also indicated in the triple combination (X2) concentration as well as in the Cr (X2) concentration when compared to the control with p-value of < 0.05. The highest % radical formation in the Cd group is shown in the X1 concentration. Based on the cytotoxicity results (Figure 4.4B), the only clear distincitive pattern is seen with the Pb group, showing a decrease in viability with an increase in concentration.

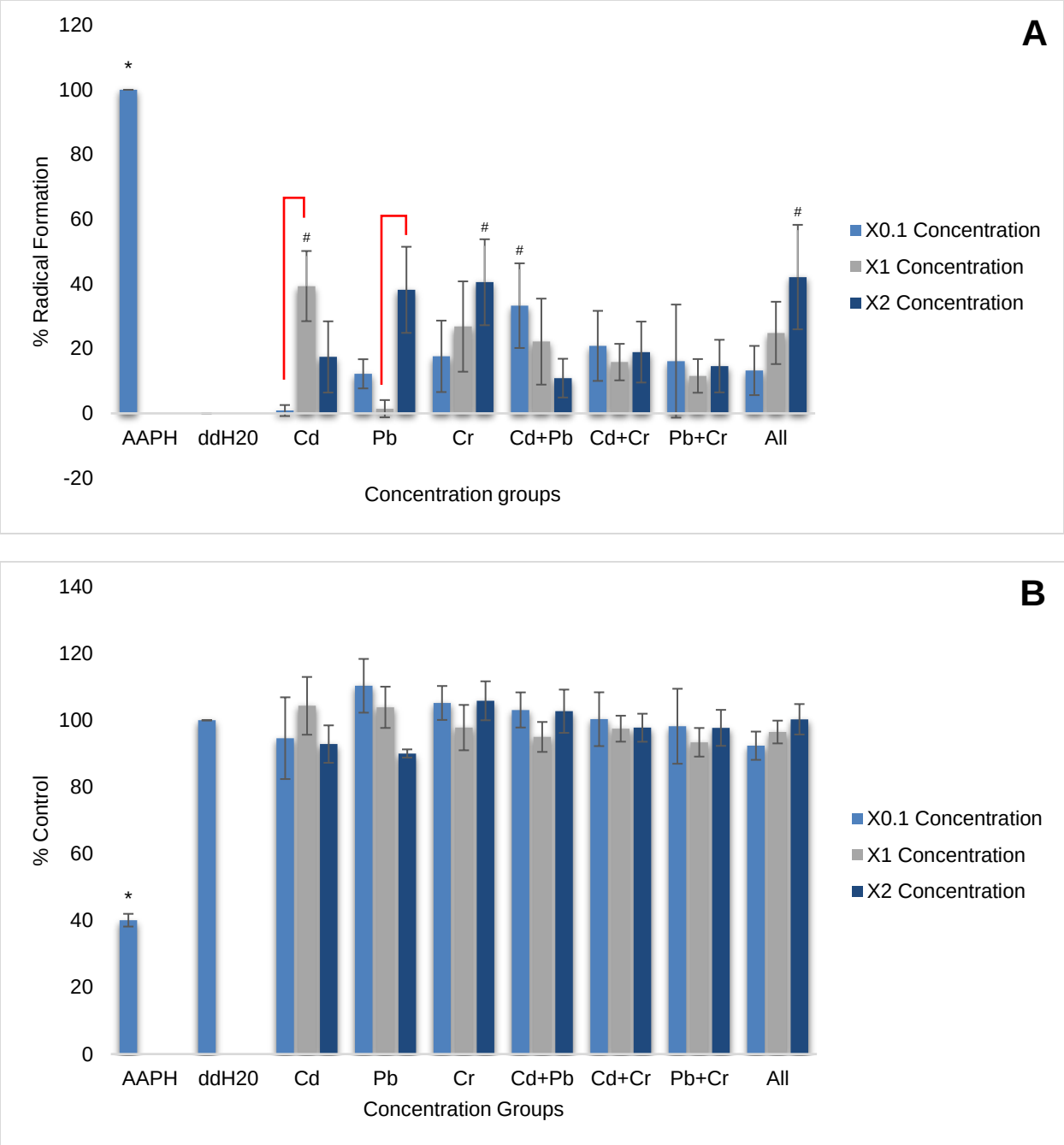
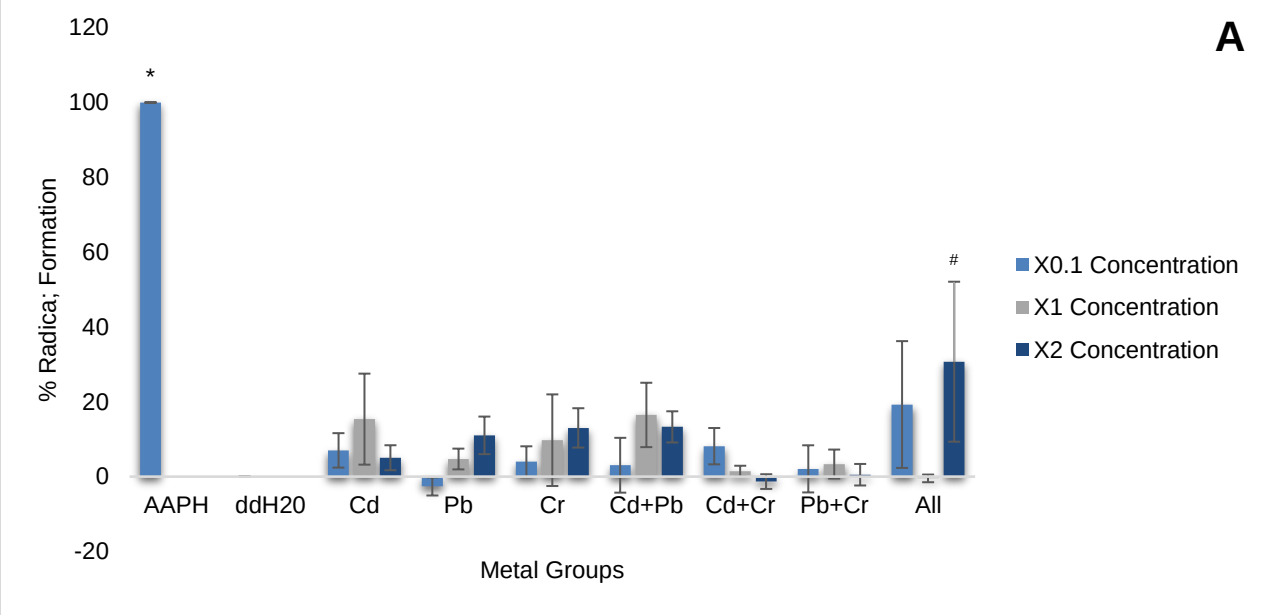


Figure 4.4 (A) - Graph indicating the % radical formation that each metal and their respective concentrations created using the DCFH-DA assay after 48h incubation. **(B) -** Graph indicating the CV assay results based on the DCFH-DA assay results for 48h incubation, with ddH_2O as the vehicle control and AAPH as the positive control. Results are expressed as mean ± SE of three independent triplicate experiments with p-value of < 0.05. * - Significance was seen between this group and every sample. . # - Significance was seen when compared to the ddH_2O group and the sample. Red line – significance was seen when compared to varying concentrations within the same metal.

4.2.3. 72 h exposure

The intracellular ROS content was assessed using the DCFH-DA assay. The standard DCFH-DA assay protocol was used. After a 72h incubation, results were read at 520 nm and expressed as % radical formation (Figure 4.5 A). The corresponding CV assay done after completion of the DCFH-DA assay was read at 630 nm and expressed as a % of the control (Figure 4.5 B). In both in-stances, ddH_2O was used as the vehicle control and AAPH as the positive control. After 72 h, the only concentration showing significant results is the triple concentration group at X2 concentration when compared to the vehicle control ddH_2O with p-value of < 0.05.



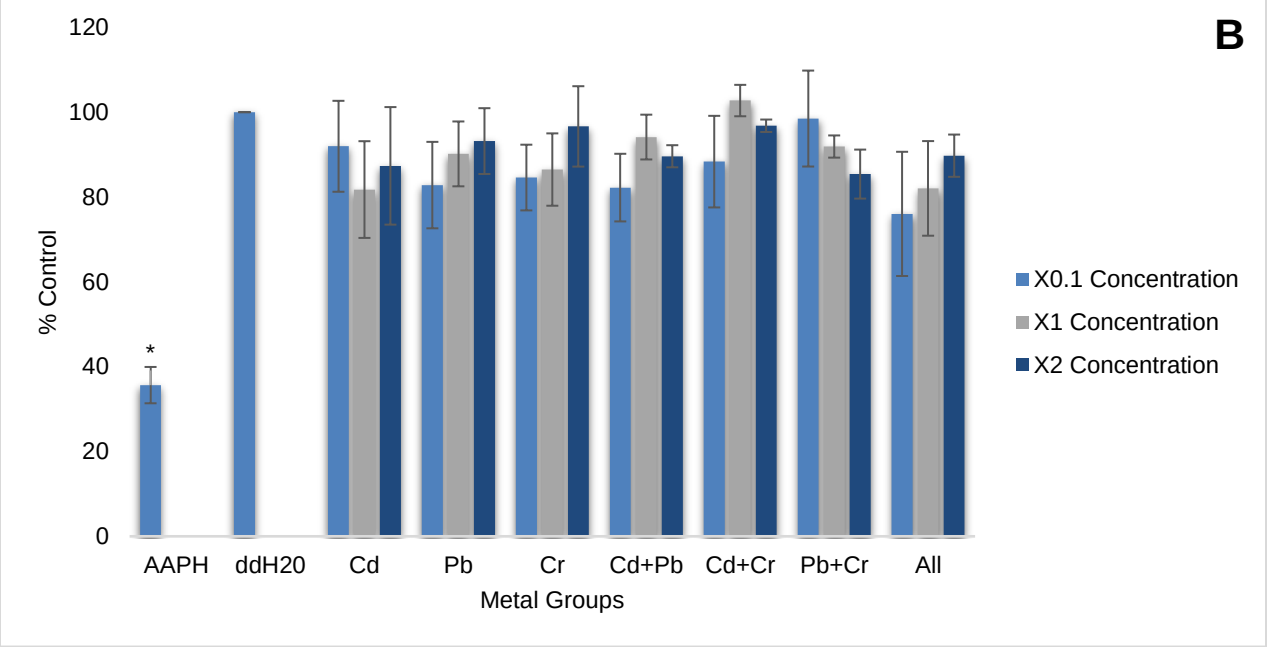


Figure 4.5 (A) - Graph indicating the % radical formation that each metal and their respective concentrations created using the DCFH-DA assay after 72h incubation. **(B) -** Graph indicating the CV assay results based on the DCFH-DA assay results for 72h incubation, with ddH_2O as the vehicle control and AAPH as the positive control. Results are expressed as mean ± SE of three independent triplicate experiments with p-value of < 0.05. * - Significance was seen between this group and every sample. # - Significance was seen when compared to the ddH_2O group and the sample.

4.3. An increase in concentration in the Pb + Cr and Cd + Pb + Cr groups caused an increase in apoptosis and necrosis.

The effect of the metal concentrations (Table 3.1) on apoptosis and necrosis was investigated using the Annexin V (Figure 4.6), and propidium iodide (Figure 4.8) to assess necrosis via flow cytometry and SEM to investigate the morphological changes. As apoptosis is classified by the presence of apoptotic bodies, and necrosis with membrane damage and intracellular component release to the outside of the cell- these were the factors taken into consideration when analysing the SEM micrographs (Table 4.2, (Figure 4.9 – 4.16).

A clear trend was seen with the Pb + Cr as well as the triple combination group with the increase of both apoptotic and necrotic cells (Figure 4.6). A significant difference was seen between the Pb + Cr group and the triple combination group when compared to the medium control with p-value of < 0.05. The flow cytometry results were analysed using Kalusa C software (Figure 4.7). Colours indicated for each cell event below corresponds with the Kalusa C analysis shown in Figure 4.7.

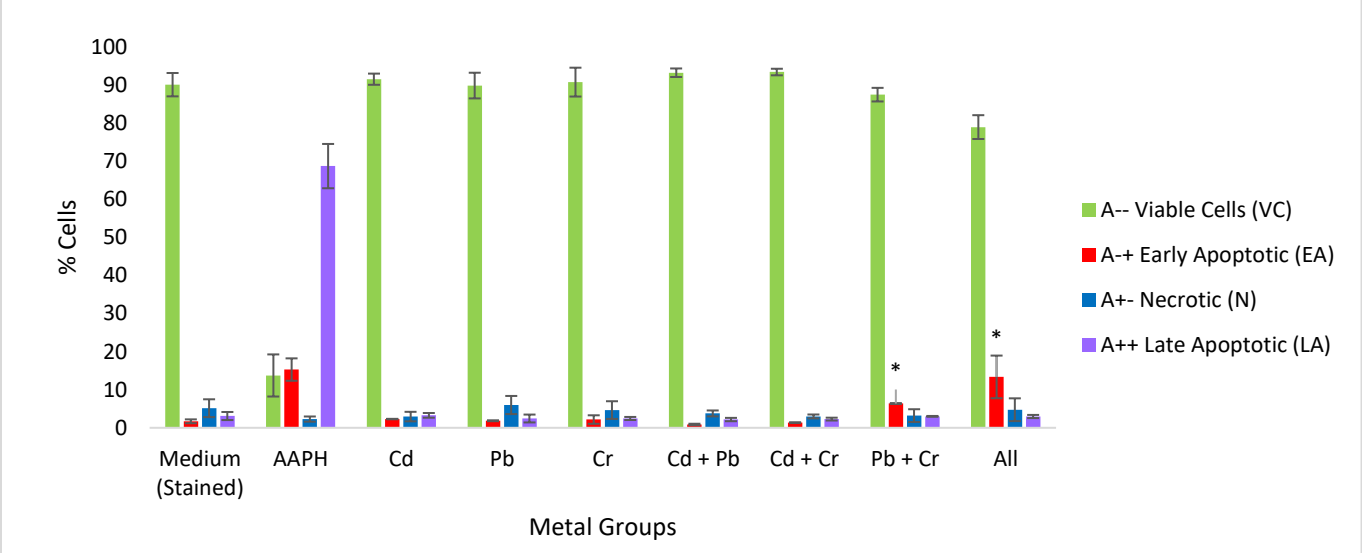


Figure 4.6- Graph indicating the Annexin V results of the X2 concentrations of metals using flow cytometry. VC- viable cells which stained negative for both Annexin V and PI, EA- Early Apoptotic, stained positive for Annexin V and negative for PI, N- necrotic, stained negative for Annexin V and positive for PI and LA- Late Apoptotic, stained positive for both Annexin V and PI. Results are expressed as mean ± SE of three independent triplicate experiments with p-value of < 0.05. * - Significance was seen when compared to the medium control.

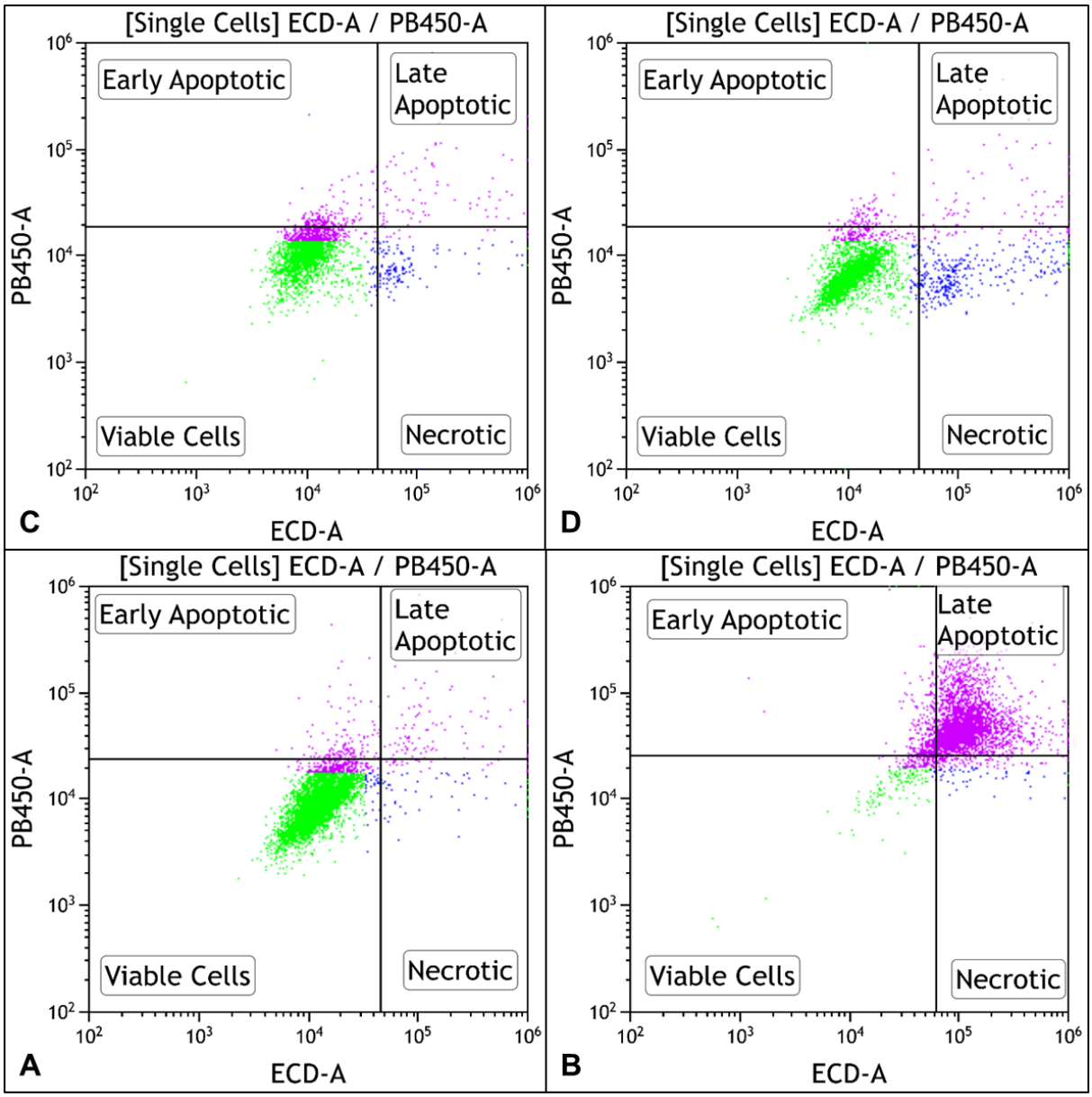


Figure 4.7- Annexin V analysis done via Kalusa C Software. Significance was seen between the Pb + Cr sample and the triple combination sample when compared to the medium control. A- Medium control, B- AAPH (1mg/ml) positive control, C- Pb + Cr Group and D - All Group.

The potential of heavy metal concentrations to induce necrosis was further analysed with the PI assay via flow cytometry. The results are indicated in Figure 4.8. An increase in the % necrosis was seen in the Cd + Cr, Pb + Cr and the triple combination group with an increase in metal concentration, with the most significant difference noted in the Pb + Cr group. This correlates with the results from the Annexin V assay.

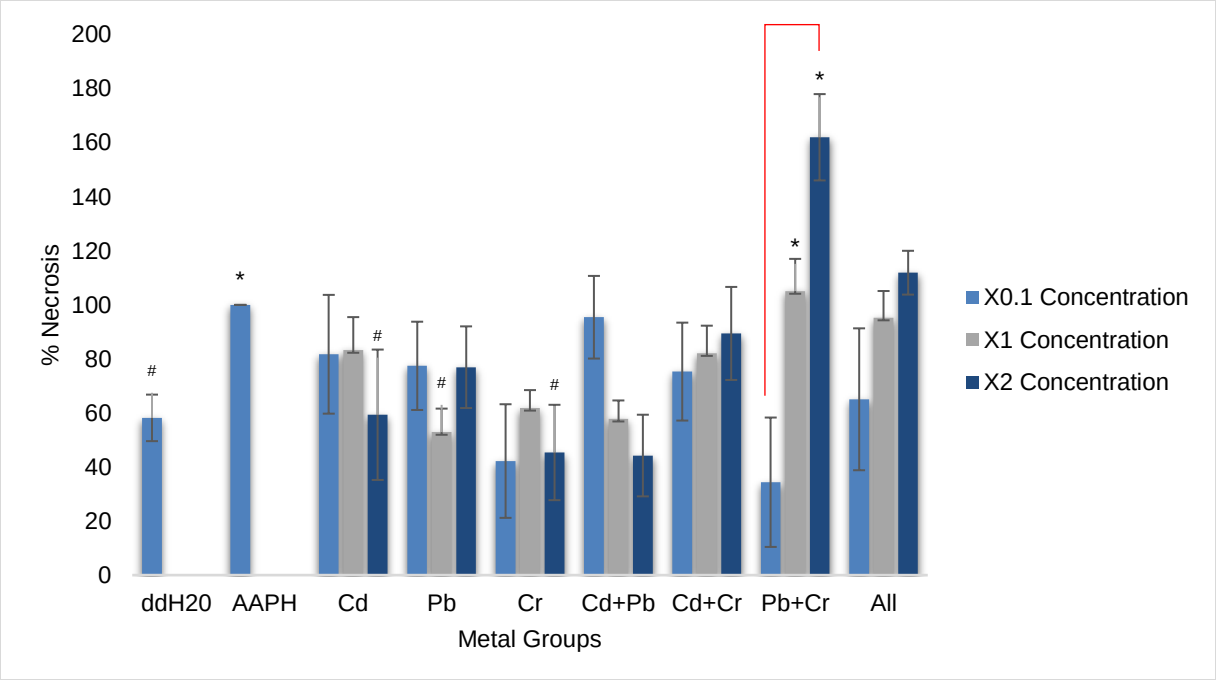


Figure 4.8- Graph indicating % Necrosis performed using the Propidium Iodide Assay with 0.1% AAPH as the positive control and ddH_20 as the vehicle control. Results are expressed as mean ± SE of three independent triplicate experiments with p-value of < 0.05. *- Significance found between this sample and ddH_20, #- significance found between this sample and Pb + Cr group. Red line – significance was seen when compared to varying concentrations within the same metal.

The morphological changes caused by the metals were investigated with SEM.

Control

The scanning electron micrographs of EA.hy926 cells from the control and exposed groups are shown in Figures 4.9 - 4.16 below. Figure 4.9 is a representative of the control samples where the varying normal morphology is shown. Some cells appeared elongated (Figure 4.9A) whereas others had a more rounded structure (Figure 4.9 B and C). A high magnification micrograph of the cell membrane is shown in Figure 4.9 D.

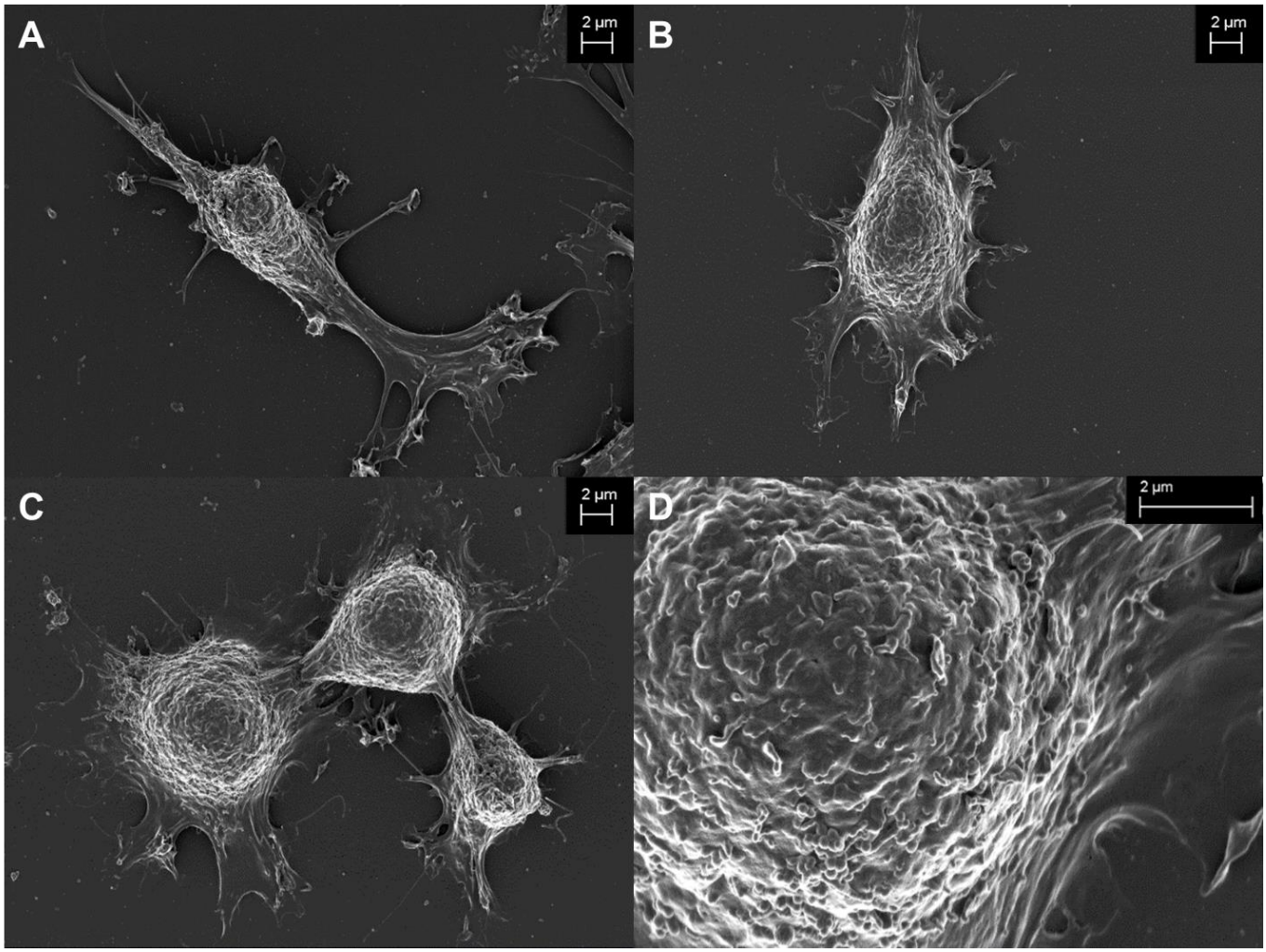


Figure 4.9 - SEM photographs of the control group treated with ddH_2O. (A-C: different morphologies of the endothelial cells D: high magnification micrograph of the surface an endothelial cell.

Cd

Figure 4.10 are representative micrographs from the Cd-exposed group. In this group some membrane damage in the form of formation of holes were observed as indicated by the white circles in 4.10 B, D and F. The presence of membrane blebbing was also seen as indicated by the red arrows in Figure 4.10 C, D, E and F, with Figure 4.10 D indicating the highest prevalence of cellular membrane blebbing.

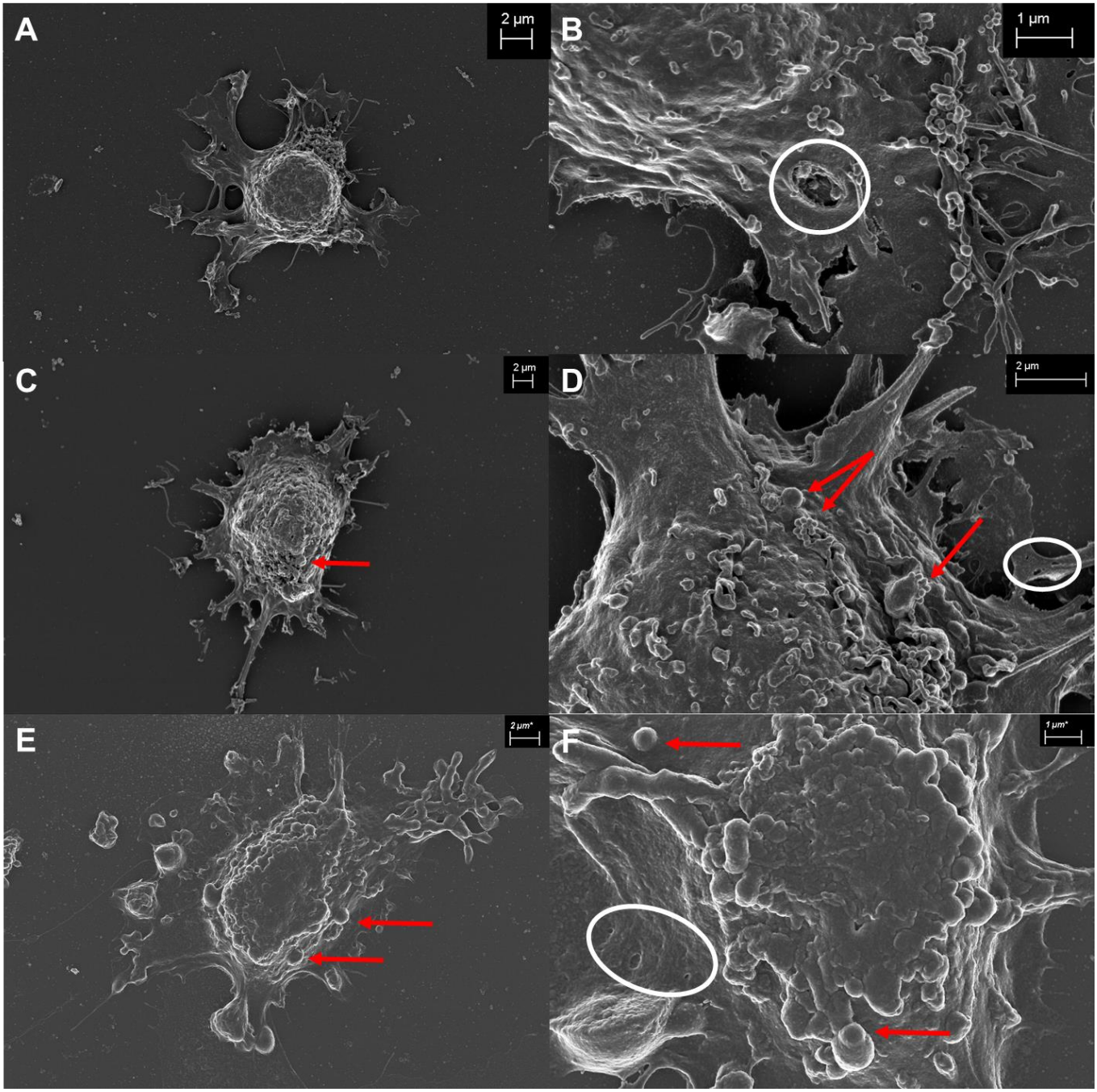


Figure 4.10- SEM micrographs of the Cd group. (A) & (B) shows the X0.1 concentration group. (C) & (D) shows the X1 concentration group. (E) & (F) shows the X2 concentration group. White Circles – membrane damage, red arrows – membrane blebbing.

Pb

Figure 4.11 are representative micrographs from the Pb-exposed group. In this group some membrane damage in the form of formation of holes were observed as indicated by the white circles in 4.11 B and F. The presence of membrane blebbing was also seen as indicated by the

red arrows in Figure 4.11 B, C, E and F, with Figure 4.11 F having the most extensive membrane blebbing.

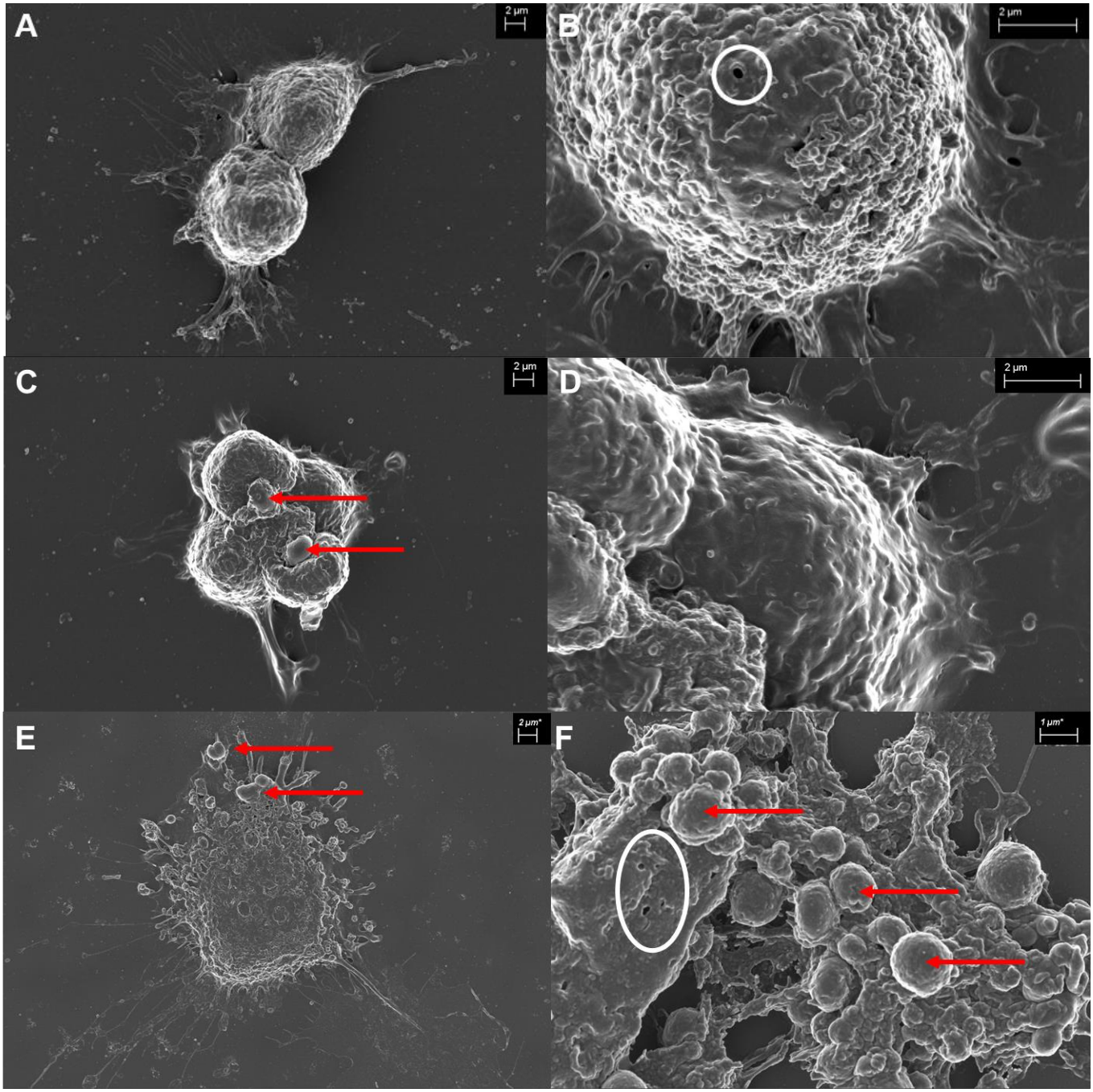


Figure 4.11- SEM micrographs of the Pb group. (A) & (B) shows the X0.1 concentration group. (C) & (D) shows the X1 concentration group. (E) & (F) shows the X2 concentration group. White Circles – membrane damage, red arrows – membrane blebbing.

Cr

Figure 4.12 are representative micrographs from the Cr-exposed group. In this group only the presence of membrane blebbing was seen as indicated by the red arrows in Figure 4.12 A, B C, D, E and F, with Figure 4.12B having the highest prevalence of membrane blebbing in this group.

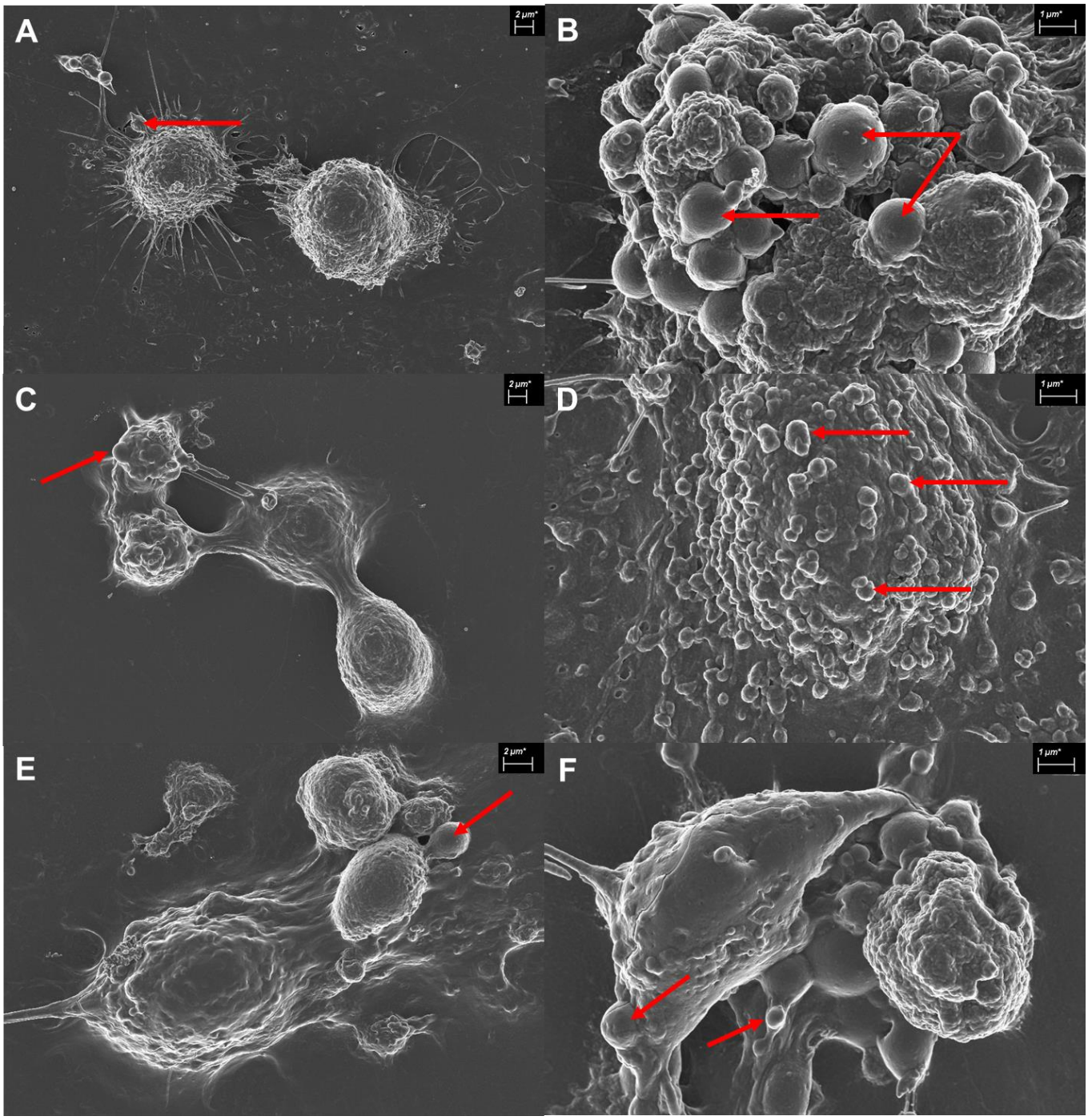
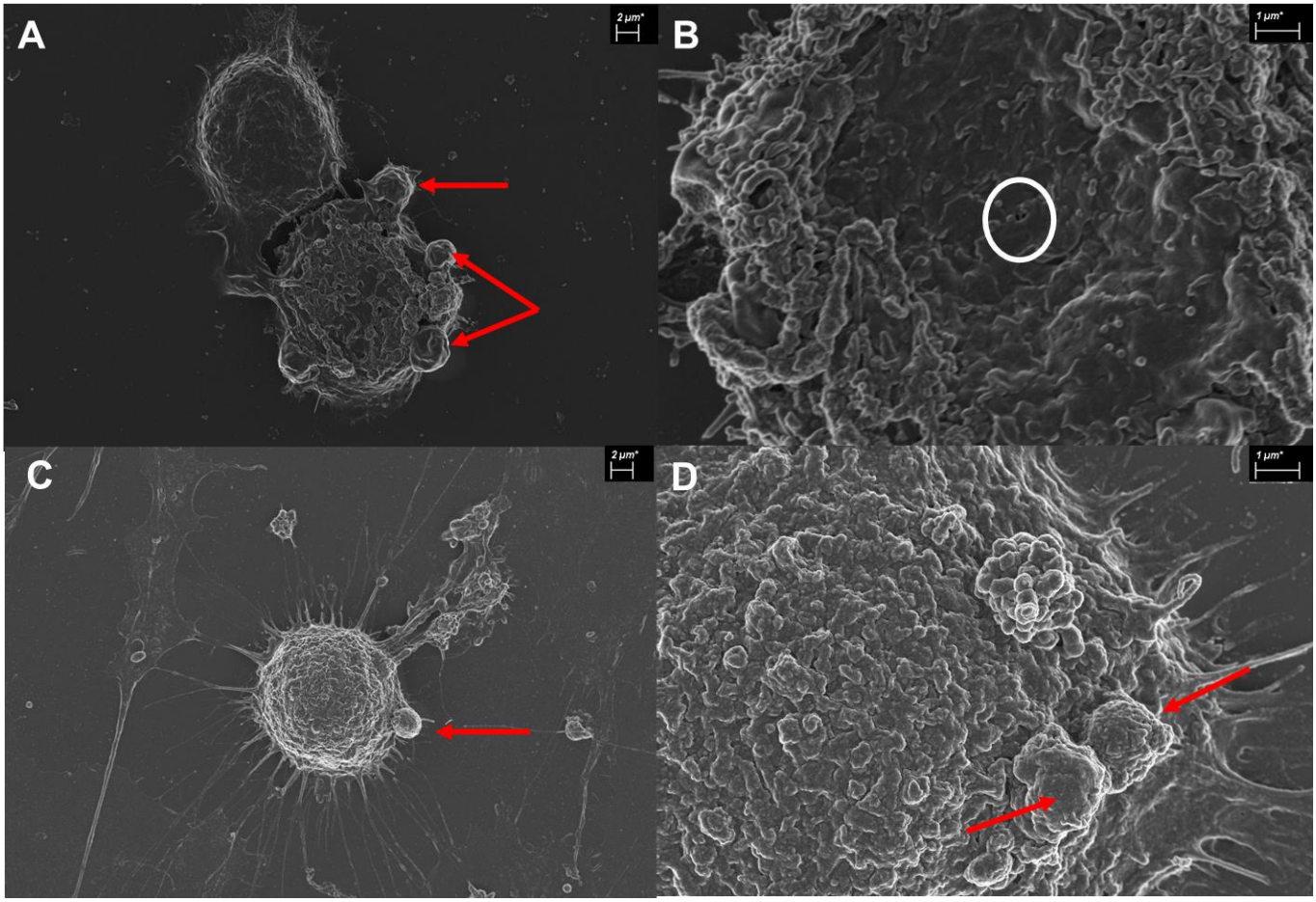


Figure 4.12- SEM micrographs of the Cr group. (A) & (B) shows the X0.1 concentration group. (C) & (D) shows the X1 concentration group. (E) & (F) shows the X2 concentration group. White Circles – membrane damage, red arrows – membrane blebbing.

Cd+ Pb

Figure 4.13 are representative micrographs from the Cd + Pb-exposed group. In this group some membrane damage in the form of formation of holes were observed as indicated by the white circles in 4.13 A, B and F, with Figure 4.13F showing a distinctive tare in the membrane. The presence of membrane blebbing was seen as indicated by the red arrows in Figure 4.13 A, B C, D, E and F, with Figure 4.13D having the highest prevalence of membrane blebbing in this group.



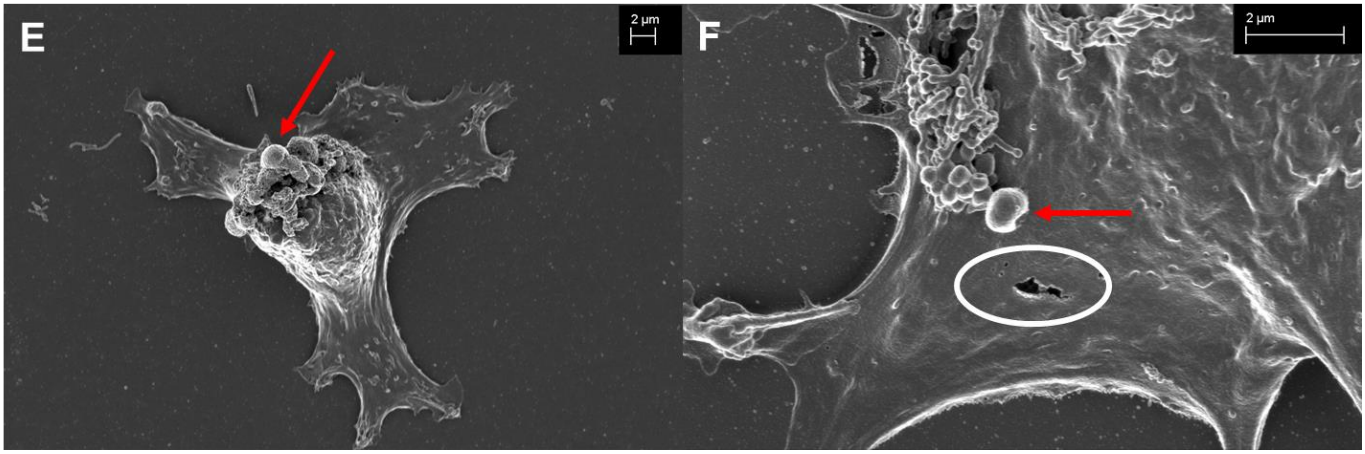
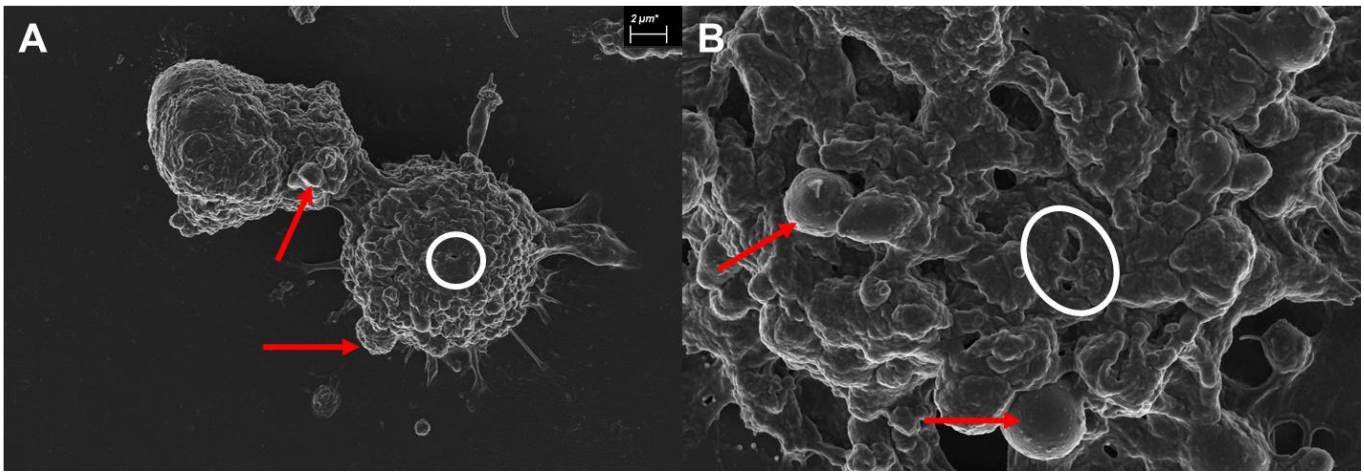


Figure 4.13- SEM micrographs of the Cd + Pb group. (A) & (B) shows the X0.1 concentration group. (C) & (D) shows the X1 concentration group. (E) & (F) shows the X2 concentration group. White Circles – membrane damage, red arrows – membrane blebbing.

Cd + Cr

Figure 4.14 are representative micrographs from the Cd + Cr-exposed group. In this group some membrane damage in the form of formation of holes were observed as indicated by the white circles in 4.14 B, C, D, E and F, with Figure 4.14F showing a distinctive tare in the membrane. The presence of membrane blebbing was seen as indicated by the red arrows in Figure 4.14 A, B C, D, E and F, with Figure 4.14D having the highest prevalence of membrane blebbing in this group.



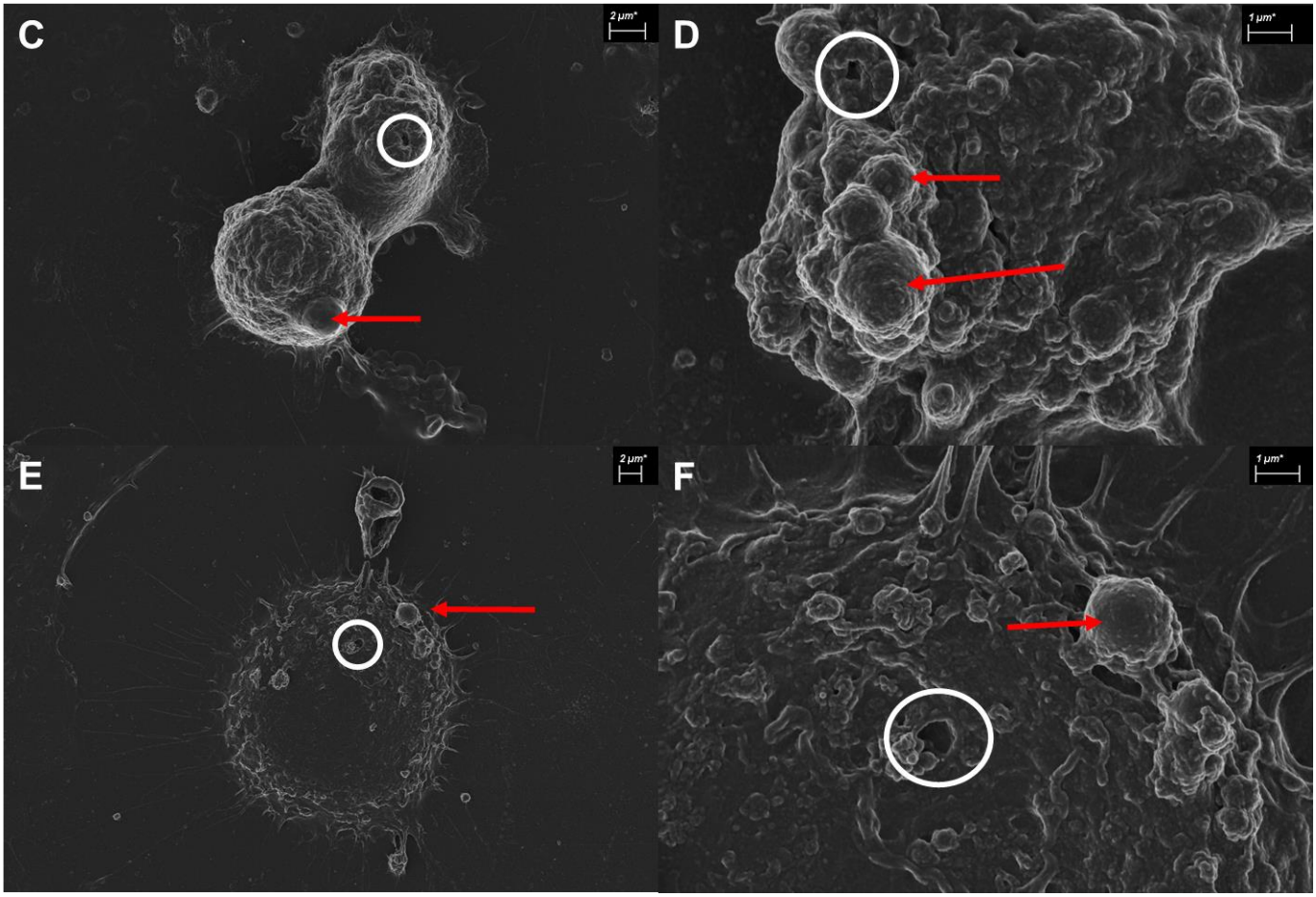


Figure 4.14 - SEM micrographs of the Cd + Cr group. (A) & (B) shows the X0.1 concentration group. (C) & (D) shows the X1 concentration group. (E) & (F) shows the X2 concentration group. White Circles – membrane damage, red arrows – membrane blebbing.

Pb + Cr

Figure 4.15 are representative micrographs from the Pb + Cr-exposed group. In this group some membrane damage in the form of formation of holes were observed as indicated by the white circles in 4.15 B, C, and D with Figure 4.15 D showing an excessive tare in the membrane. The presence of membrane blebbing was seen as indicated by the red arrows in Figure 4.15 A, B, E and F, with Figure 4.15F having the highest prevalence of membrane blebbing in this group.

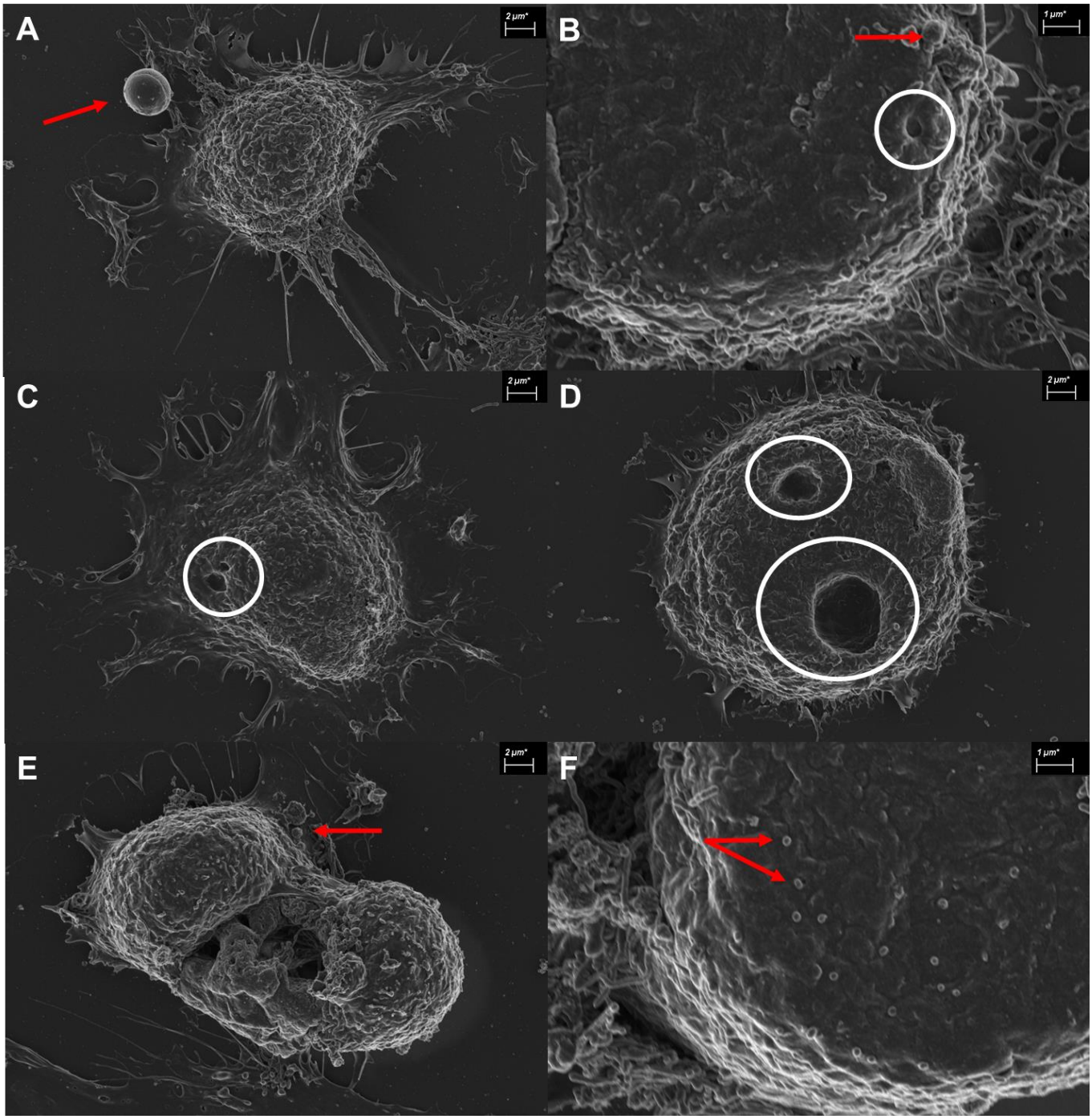
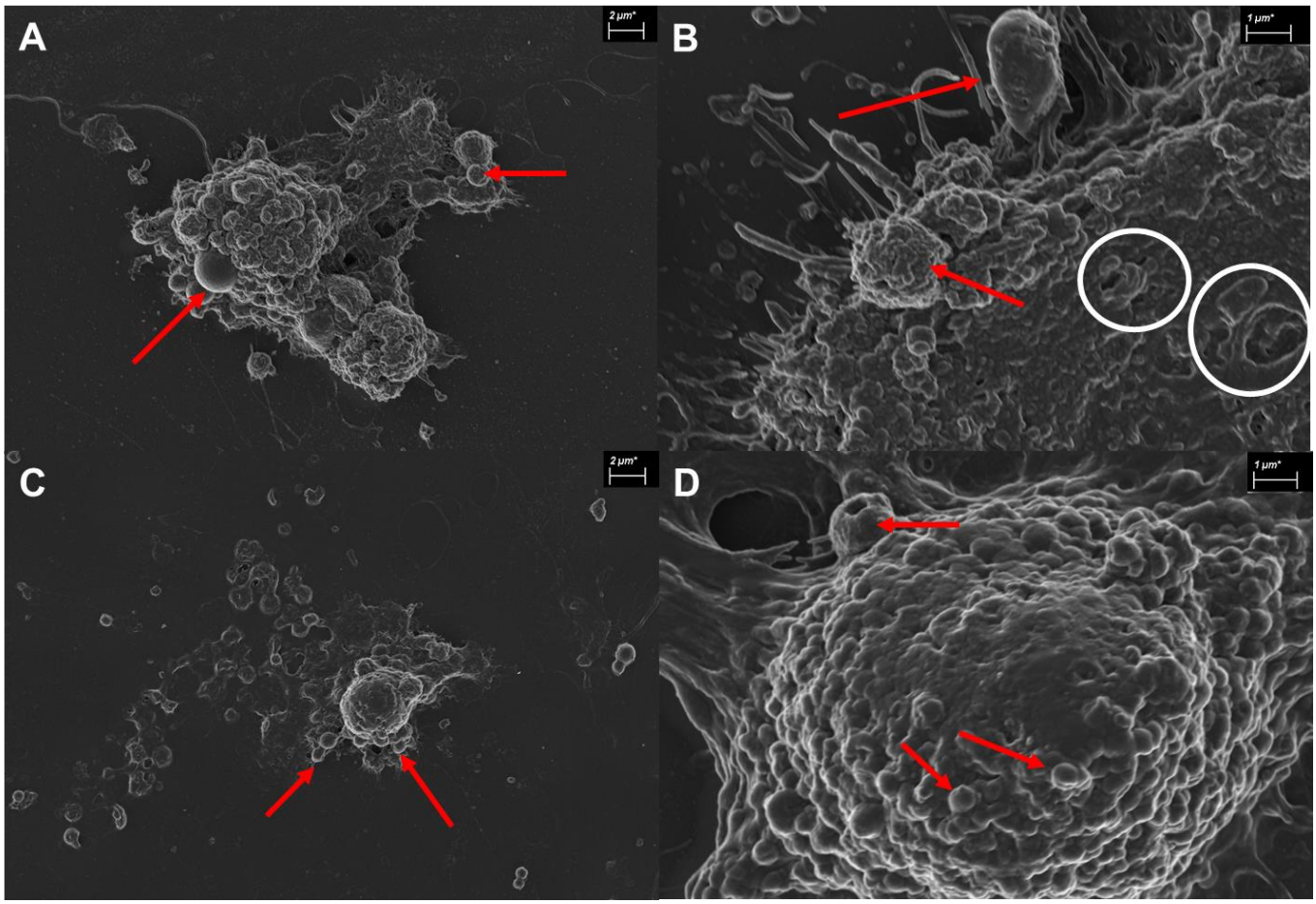


Figure 4.15- SEM micrographs of the Pb + Cr group. (A) & (B) shows the X0.1 concentration group. (C) & (D) shows the X1 concentration group. (E) & (F) shows the X2 concentration group. White Circles – membrane damage, red arrows – membrane blebbing.

Cd + Pb + Cr

Figure 4.16 are representative micrographs from the Cd + Pb + Cr-exposed group. In this group some membrane damage in the form of formation of holes were observed as indicated by the white circles in 4.16 B and F, with Figure 4.14B showing a distinctive tare in the membrane. The presence of membrane blebbing was seen as indicated by the red arrows in Figure 4.16 A, B C, D, E and F, with Figure 4.14F having the highest prevalence of membrane blebbing in this group.



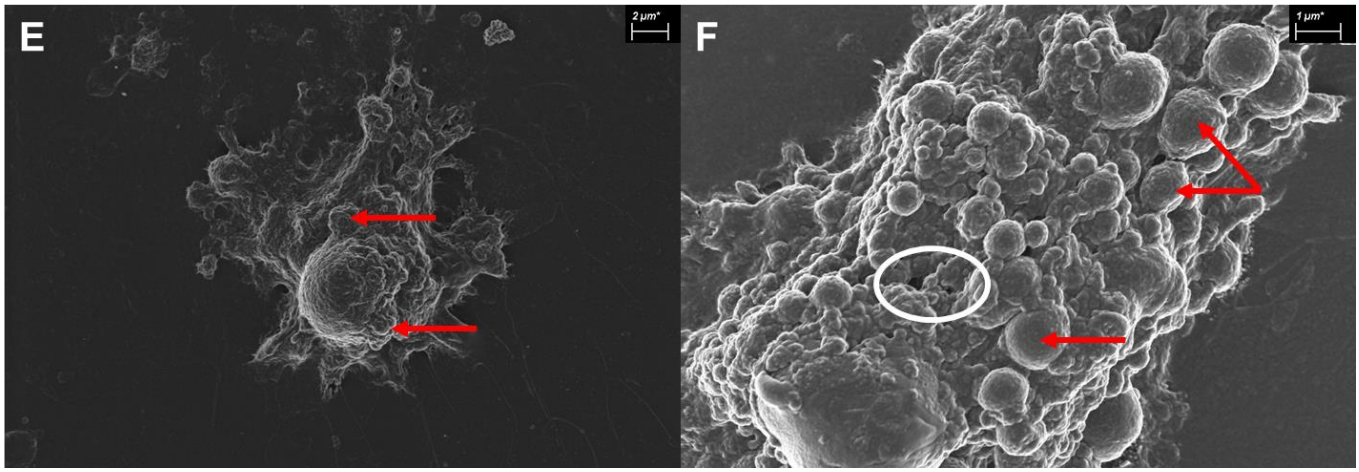


Figure 4.16- SEM micrographs of the Cd + Pb + Cr group. (A) & (B) shows the X0.1 concentration group. (C) & (D) shows the X1 concentration group. (E) & (F) shows the X2 concentration group. White Circles – membrane damage, red arrows – membrane blebbing.

Table 4.2 below summarizes the results obtained according to the prevalence of membrane blebbing and damage to the cell membrane as compared to the control.

Table 4.1- Table summarizing the prevalence of blebs and cell membrane damage from the SEM analysis of each metal group

Group	Concentration	Prevalence of blebs	Prevalence of damage to the cell membrane
Control	-	-	-
Cd	X0.1	-	+
	X1	+	+
	X2	++	+
Pb	X0.1	+	+
	X1	++	-
	X2	+++	++
Cr	X0.1	++	-
	X1	+++	-
	X2	++	-

Cd + Pb	X0.1	+	+
	X1	++	-
	X2	+	++
Cd + Cr	X0.1	+	+
	X1	++	+
	X2	+	++
Pb + Cr	X0.1	+	+
	X1	+	++
	X2	++	+++
Cd + Pb + Cr	X0.1	++	+
	X1	++	-
	X2	+++	++

None (-), Mild (+), Moderate (++), Severe (+++)

In almost all of the metal-exposed groups, cellular membrane blebbing was seen with the highest prevalence seen in the Pb, Cr and triple combination group. The presence of membrane damage in the form of formation of holes was most evident in the Pb + Cr group. Membrane damage is a clear indication of necrosis, and is consistent with the propidium iodide assay (Figure 4.8) & Annexin V assay (Figure 4.6) results.

Chapter 5: Discussion

The term heavy metal is a generic term used to describe any metallic element, such as metalloids or metals that are stable and have relatively higher densities when compared to water (59). Metals are essential to several physiological processes within the human body, such as forming part of constituents of several key enzymes, and some are co-factors for many oxidation–reduction reactions (56). However, it also has the potential to affect the health of individuals when the concentration is not within the physiological favourable range (4). Although heavy metals are considered as trace elements in a variety of environmental conditions, their physical and chemical properties, such as their charge, oxidation state, geometry and solubility influence their mobility and bioavailability, giving them a high level of reactivity when placed in biological systems (59).

Cigarettes are made up of tobacco, paper and numerous additives. Several of these additives contain environmental contaminants and can cause exposure to heavy metals such as mercury, cadmium and lead (4). A recent study conducted in 2022, revealed that tobacco smoke contains a total of 83 carcinogens, of which 37 are in unburned tobacco and 80 in tobacco smoke (60).

Respiratory tract infections and complications are common causes of morbidity and mortality among smokers, this is due to the fact that the lungs are the first organ to be exposed to cigarette smoke during inhalation (61). Cigarette smoke has been linked to having the potential to increase the susceptibility of the lungs to lung infections and acute respiratory disease syndrome (ARDS) by increasing the alveolar-capillary barrier permeability and inflammation in humans (61). The effect of cigarette smoke on lung endothelial cells was investigated by Sharma *et al*., (62), and the results indicated that cigarette smoke activates the lung endothelium and causes inflammatory cell accumulation. The authors also found that cigarette smoke exposure induces necrosis of both bronchial epithelial cells and neutrophils (62). Both the above mentioned studies concluded that cigarette smoke exposure causes endothelial cell dysfunction directly and is associated with vascular remodelling and is the cause of vasoconstriction commonly observed in smokers (61, 62). A study conducted by Noronha *et al*., (63) also tested the effect of cigarette smoke on endothelial cells. The findings of this study revealed that cigarette smoke is involved in endothelial injury and it is mediated by the oxidative burden imposed by the free radicals that are present in cigarette smoke (63).

Cadmium is a natural occurring element found primarily in the earth's crust and is commonly found in conjunction with another elements predominantly O_2 or Cl_2 (64). When smoking, Cd is

converted to cadmium oxide, which is inhaled. Almost 10% of the Cd is then deposited in the lungs, and 20 to 50% is transported into circulation (5). The fact that the human body is unable to excrete Cd makes the health effects of Cd- exposure more aggravated (30). Cadmium-induced toxicity has been widely studied and it has been shown that Cd can induce apoptosis in various cell types. Growing evidence suggests that elevated serum levels of Cd correlate with the risk of vascular diseases and endothelial cell dysfunction (30, 31). Lead is known to have no biological or physiological role in the body (26), therefore, very small dosages of Pb can be considered toxic when introduced into the system. Chromium helps control whole body metabolism, via the breakdown of fats and carbohydrates and it stimulates fatty acid and cholesterol synthesis (2). Exposure to increased amounts of Cr compounds could lead to the development of ulcers, namely nasal septal ulcers which are commonly seen in chromate workers (24). Exposure to vastly larger amounts of Cr compounds could lead to the inhibition of erythrocyte glutathione reductase, which then inhibits the ability to reduce methaemoglobin to haemoglobin (24). Both *in vivo* and *in vitro tests* have indicated that Cr compounds cause DNA damage which in turn leads to the formation of chromosomal aberrations, DNA adducts, alterations in transcription of DNA and replication sister chromatid exchanges (23).

Heavy metals are accountable for various physiological, biochemical and morphological disruptions in human cellular processes (65). The generation of ROS in cells are usually accountable for redox-active metals such as Fe and Cu, however, heavy metals such as Pb, Cd, Ni and Zn cannot generate ROS directly through interference in biological redox reactions such as the Fenton reaction in the body. These metals in turn induce ROS generation via a number of indirect mechanisms namely; displacement of essential cations from specific binding sites of enzymes, inhibition of enzymatic functioning through the bio affinity of heavy metals for –SH groups and by stimulation of NADPH oxidases (65). The production of ROS in endothelial cells has specifically been linked to cardiovascular disease states (66).

A study conducted by Yaprak *et al.,* (6), determined which metals are the most predominant in the blood of chronic smokers, as well as their exact metal concentrations. Their results indicated that the three most prevalent metals in cigarette smoke was Cd at a concentration of 0.21 µg/L, Pb at a concentration of 4.1 µg/L and Cr at a concentration of 224 µg/L (6). Based on these results, the current study aimed to investigate the effects of Cd, Pb and Cr, alone and in combination on the endothelial cell line, EA.hy926 by determining the cytotoxicity, ability of these metals to produce ROS, to test the induction of apoptosis and necrosis and to assess the morphological changes induced after heavy metal exposure.

For all the concentrations of the metals tested, for both the singular and combination groups, no cytotoxic abilities were seen in any of the groups in both the MTT and CV assays. A study conducted by Kopp *et al.*, (59), investigated the genotoxicity of 11 heavy metals, of which $CdCl_2$ and $PbCl_2$ was amongst the samples tested on HepG2 cells. The authors determined the lowest observed adverse effect concentration (LOAEC) for each metal and reported it to be 25 µM for $CdCl_2$ and 100 µM for $PbCl_2$ (59). Another study tested the adverse effects of $CdCl_2$, $MeHgCl_2$ and $PbCl_2$ through a variety of equal molar concentrations (0, 15, 30, 60, 125, 250 µM) (67). These results were further substantiated by Lozi *et al* (68). Cytotoxicity was seen at concentrations of 15 µM and above for each heavy metal. Based on the metal concentrations analysed (Table 3.1), the highest concentration of Cd analysed was 2.29×10^{-3} µM, and the highest concentration of Pb analysed was 0.029 µM, which is much lower than the LOAEC. This might be a possible reason for the results obtained in the current study where no cytotoxicity was observed in any of the concentrations tested.

Reactive oxygen species generation with an increase in metal concentration was specifically seen in the single metal groups as well as in the triple combination group. Similar results were seen in a study conducted by Das *et al* (69)., were the effect of ambient fine particular matter associated metals on the lung carcinoma epithelial cell line, A549 was tested. The results indicated a strong correlation between Cd, Ni and Cr exposure and subsequent ROS production (69). Fu *et al.,* (70), further concluded that heavy metals tend to have an accumulative effect of ROS production after a longer period of time, as an accumulative effect is seen after 24h. This was substantiated by the more constant results at the 48 h compared to the 24 h reading of the DCFH-DA assays in this study.

The apoptotic and necrotic effects of the various metal concentrations alone and in combination was assessed using the Annexin V assay with flow cytometry. Results indicated a significant number of cells in the early apoptotic phase for the Pb + Cr group, as well as for the triple combination group. To substantiate the results obtained from the Annexin V assay, a propidium iodide assay was conducted to determine the percentage necrosis formation in all the metal groups at all concentrations tested. Results indicated a significant increase in necrosis formation in the Pb + Cr and triple combination groups. An increase in the percentage necrosis was also seen in the Cd + Cr group, however this was not significant. Chukwuebuka *et al.,* (68), tested the cytotoxicity and mechanisms of cell death induced by heavy metals Cd, Pb, As and Cr on basophilic leukaemia (RBL-2H3) cells. Results indicated an additive effect in both the Pb+ Cd and in Pb+ Cr groups. The study also further revealed that Pb, Cd, AsO_4 and Cr induced significant

cell death by apoptosis in the RBL-2H3 cell line with a highly significant necrotic cell death observed in the Pb +Cr group specifically (68).

A study conducted by Martínez-Nava *et al* (71)., studied the effect of Cd on the concentration of essential metals in a human chondrocytes. A 3D culture of human chondrocytes was phenotyped using the Western blot technique and thereafter structurally evaluated with histological staining. The samples were exposed to 1, 5, and 10 µM of $CdCl_2$ for 12h. The concentrations of essential metals Fe, Mn, Zn, Cr and Ni was quantified through plasma mass spectrometry. The results showed that Cd exposure along with the same concentration of essential metals such as Mn, Fe, Ni, Zn and Cr showed less cytotoxicity than when the metal concentrations were exposed alone. This showed that Cd could possibly have an antagonistic role when combined with essential metals (71). The results of this study could possibly explain why there was no potentiating effect in the Cd + Cr group.

Analysis of the micrographs of the different metal groups obtained with SEM revealed the presence of membrane damage in the form of small tears as well as cellular membrane blebbing. A dose-dependent reaction was seen in almost each metal group, with the X2 concentrations having the most morphological changes present. All of the tested metal groups had membrane blebbing present at the X2 concentration, whereas only the Pb and combination groups indicated membrane damage. A similar study was conducted by Kakano *et al* (72)., to investigate differential acute lung cytotoxicity caused by heavy metals using a primary culture of alveolar type II cells. Results of both cytotoxicity and SEM analyses, indicated a dose-dependent relationship in each metal group tested (Cd, Pb, Hg and Ni) and significant differences in morphology was noted in the cell membrane integrity. The authors concluded that high toxicity was observed in the Cd and Hg groups along with moderate toxicity observed in the Pb and Ni groups (72). A study conducted by Sa *et al* (73)., investigated the association between heavy metal exposure and cancer in renal cells. Using both SEM, Transmission Electron Microscopy (TEM) and X-Ray microanalysis, the results obtained indicated that with increased levels of Cr, increased changes in cellular morphology was observed (73).Similar results were shown by Trabelisi *et al* (74)., where the cytotoxic and genotoxic capabilities of Cd was tested on human larynx cells, the SQ20B cells were exposed to 25 and 50 µM Cd for 48 and 72 h. Results indicated a dose-dependent decrease in cell viability with an increase in Cd concentration, specifically in the 48h readings. Interestingly, Cd exposed cells showed normal cell cycle events at the exposed concentrations, indicating that Cd does not have an effect on cell cycle events. Mitochondria alterations were

seen with TEM analysis, which substantiated the results obtained from the MTT assay to determine cytotoxicity.

Chapter 6: Conclusion and Future Perspectives

Exposure of endothelial cells (EA.hy926) to Cd, Pb and Cr alone and in combination at three different concentrations (X0.1, X1 and X2) indicated no cytotoxic effect in both the MTT and CV assays. Regarding the percentage radical formation, at the 24 h reading Cd alone showed an increase with an increase in metal concentration, with Pb alone showing the greatest percentage radical formation for the X1 concentration group. At the 48 h reading, Cr alone as well as the triple combination group showed an increase in percentage radical formation with an increase in metal concentration with Cd in turn, showing the highest percentage radical formation in the X1 concentration and Pb at the X2 concentration. After 72 h, both Cd and Pb alone showed a gradual increase in percentage radical formation with an increase in concentration, with Cd showing the highest increase in the X1 concentration. Apoptosis and necrosis were seen at increased concentrations of the Pb + Cr and triple combination groups with flow cytometry analysis, and these results were further substantiated with the increase in percentage necrosis for the Cd + Cr, Pb + Cr and triple combination groups. Morphological changes were observed with SEM, with almost all of the X2 concentrations of metals showing either damage to the cell membrane, cell blebs present or a combination of the two. As endothelial cells have a protective function in the human body, cigarette smoke diminishes its protective ability through both disruption of cellular processes and morphology. Extensive research has been done on pulmonary endothelial cells and the link that exists between excessive cigarette smoke exposure and necrotic endothelial cells. With this considered, although no cytotoxicity was observed at the concentrations tested, changes on ultrastructural level are present and should be further investigated.

For future studies, higher concentrations of these metals should be investigated as an increase in percentage radical formation, apoptosis and necrosis were observed at the X2 concentrations.

Scanning electron microscopy was used in this study where only the surface of cells were scanned. In future, transmission electron microscopy can be done to investigate the intracellular organelles, specifically the mitochondria and the nucleus to possibly identify changes related to the necrosis pathway. Another limitation is the use of a single exposure dosage of each metal and further studies can investigate a dosage effect of various metals on the endothelial cells.

Chapter 7: References

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Chapter 8: Appendices

8.1. Ethical clearance certificates

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences

Institution: The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.

- FWA 00002567, Approved dd 22 May 2002 and Expires 03/20/2022.
- IORG #: IORG0001762 OMB No. 0990-0279 Approved for use through February 28, 2022 and Expires: 03/04/2023.

Faculty of Health Sciences **Research Ethics Committee**

15 July 2021

Approval Certificate
New Application

Dear Miss L van Strijp

Ethics Reference No.: 348/2021
Title: Investigating the effect of the heavy metals cadmium, chromium and lead, alone and in combination on an endothelial cell line

The **New Application** as supported by documents received between 2021-06-08 and 2021-07-14 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on 2021-07-14 as resolved by its quorate meeting.

Please note the following about your ethics approval:

- Ethics Approval is valid for 1 year and needs to be renewed annually by 2022-07-15.
- Please remember to use your protocol number (348/2021) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

On behalf of the FHS REC, Dr R Sommers
MBChB, MMed (Int), MPharmMed, PhD
Deputy Chairperson *of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria*

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health)

Research Ethics Committee
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Fakulteit Gesondheidswetenskappe
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UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences

Institution: The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.

- FWA 00002567, Approved dd 18 March 2022 and Expires 18 March 2027.
- IORG #: IORG0001762 OMB No. 0990-0278 Approved for use through August 31, 2023.

Faculty of Health Sciences **Research Ethics Committee**

15 June 2022

Approval Certificate
Annual Renewal

Dear Miss L van Strijp,

Ethics Reference No.: 348/2021 – Line 1
Title: Investigating the effect of the heavy metals cadmium, chromium and lead, alone and in combination on an endothelial cell line

The **Annual Renewal** as supported by documents received between 2022-05-18 and 2022-06-15 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on 2022-06-15 as resolved by its quorate meeting.

Please note the following about your ethics approval:

- Renewal of ethics approval is valid for 1 year, subsequent annual renewal will become due on 2023-06-15.
- Please remember to use your protocol number (348/2021) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

On behalf of the FHS REC, Dr R Sommers
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Fakulteit Gesondheidswetenskappe
Lefapha la Disaense tša Maphelo

8.2. Originality report

DECLARATION OF ORIGINALITY

UNIVERSITY OF PRETORIA

The Department of Anatomy places great emphasis upon integrity and ethical conduct in the preparation of all written work submitted for academic evaluation.

Academics teach you about referencing techniques and how to avoid plagiarism; it is your responsibility to act on this knowledge. If you are at any stage uncertain as to what is required, you should speak to your lecturer before any written work is submitted.

You are guilty of plagiarism if you copy something from another author's work (e.g. a book, an article or a website) without acknowledging the source and pass it off as your own. In effect you are stealing something that belongs to someone else. This is not only the case when you copy work word-for-word (verbatim) but also when you submit someone else's work in a slightly altered form (paraphrase) or use a line of argument without acknowledging it.

Students who commit plagiarism will not be given any credit for plagiarised work. The matter may also be referred to the Disciplinary Committee (Students) for a ruling. Plagiarism is regarded as a serious contravention of the University's rules and can lead to expulsion from the University.

The declaration which follows must accompany all written work submitted while you are a student of the Department of Anatomy No written work will be accepted unless the declaration has been completed and submitted.

Full names and surname of student: Leigh-Ann van Strijp

Student number: 15018033

Topic of work: Investigating the effect of the heavy metals cadmium, chromium and lead, alone and in combination on an endothelial cell line.

Declaration

1. I understand what plagiarism is and am aware of the University's policy in this regard.

2. I declare that this Dissertation (e.g. essay, report, project, assignment, dissertation, thesis, etc) is my own original work. Where other people's work has been used (either from a printed source, Internet or any other source), this has been properly acknowledged and referenced in accordance with departmental requirements.

SIGNATURE 29.09.2022 **DATE**