





































































































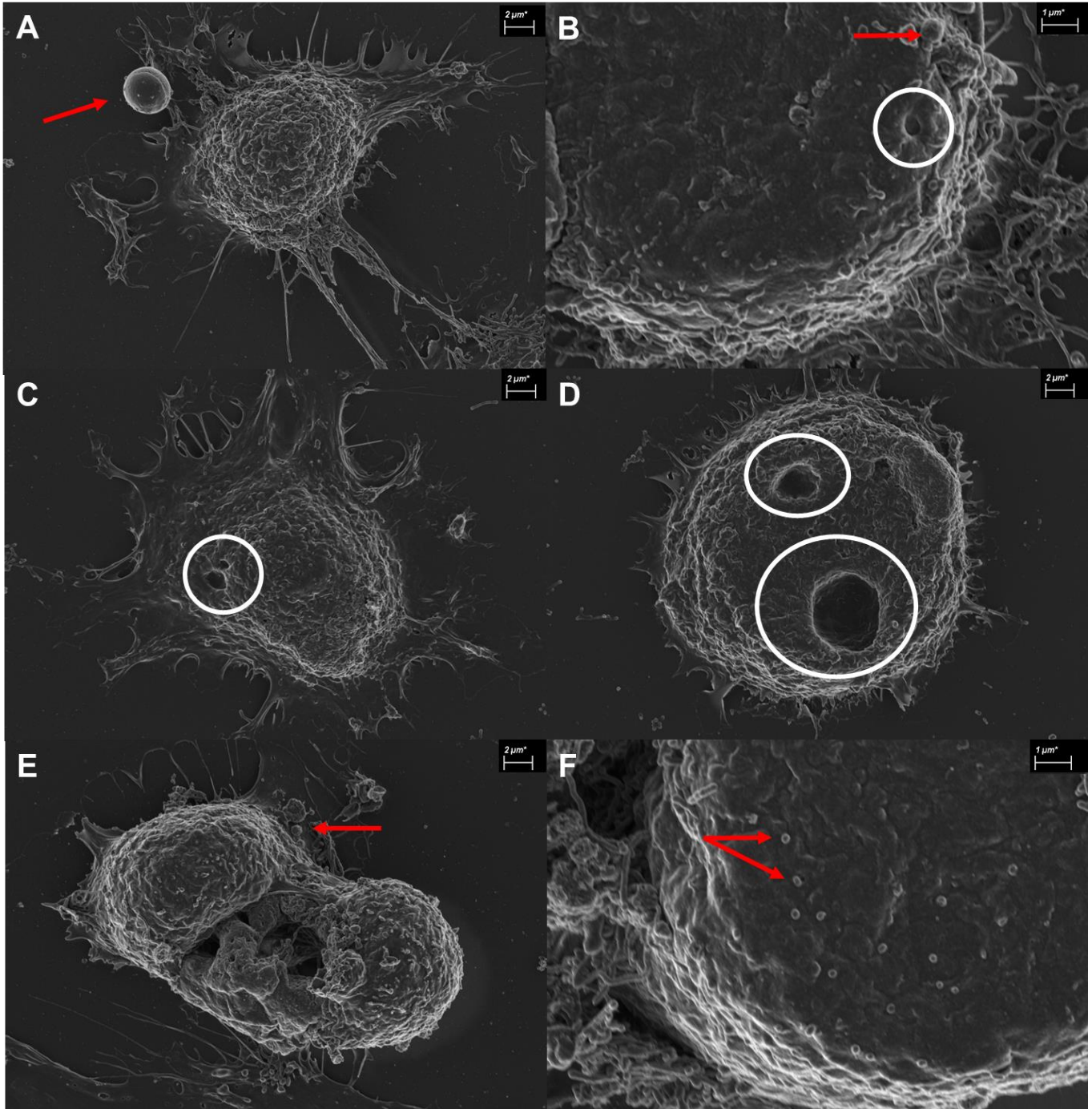






## Pb + Cr

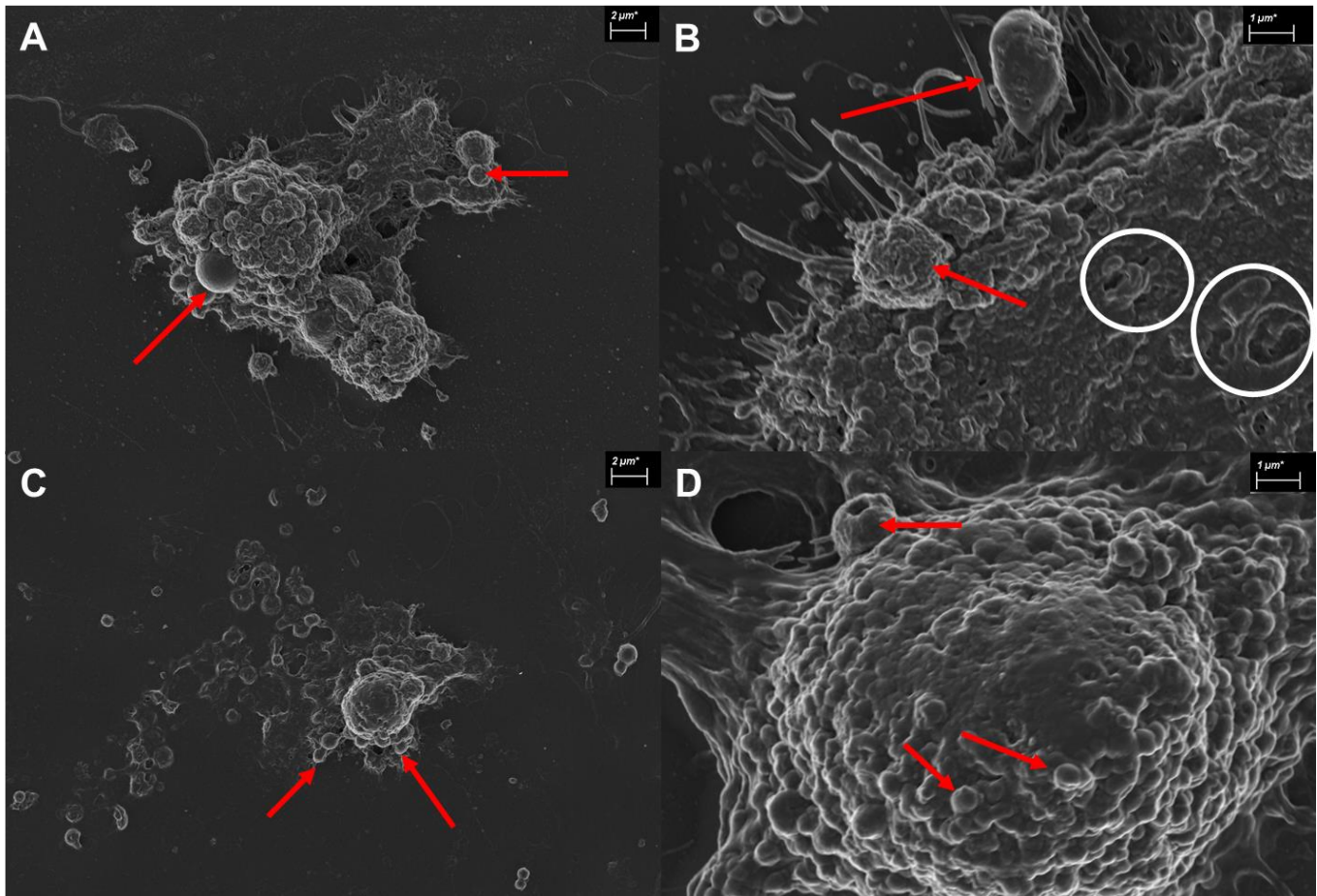
Figure 4.15 are representative micrographs from the Pb + Cr-exposed group. In this group some membrane damage in the form of formation of holes were observed as indicated by the white circles in 4.15 B, C, and D with Figure 4.15 D showing an excessive tare in the membrane. The presence of membrane blebbing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

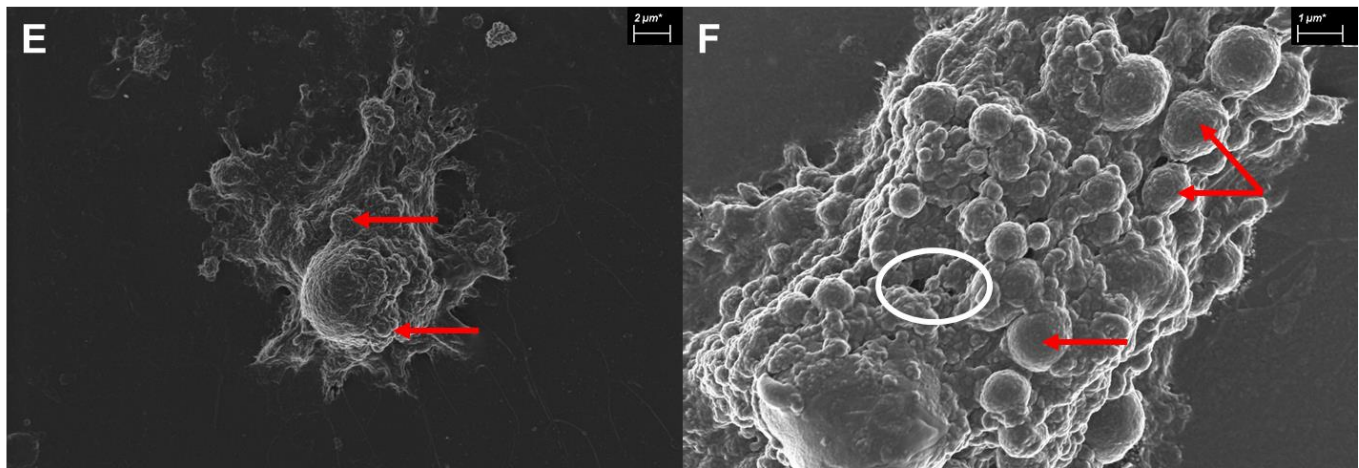


**Figure 4.15-** SEM micrographs of the Pb + Cr group. (A) & (B) shows the X0.1 concentration group. (C) & (D) shows the X1 concentration group. (E) & (F) shows the X2 concentration group. White Circles – membrane damage, red arrows – membrane blebbing.

### **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	++	-

<b>Cd + Pb</b>	X0.1	+	+
	X1	++	-
	X2	+	++
<b>Cd + Cr</b>	X0.1	+	+
	X1	++	+
	X2	+	++
<b>Pb + Cr</b>	X0.1	+	+
	X1	+	++
	X2	++	+++
<b>Cd + Pb + Cr</b>	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

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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<sub>2</sub> or Cl<sub>2</sub> (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<sub>2</sub> and PbCl<sub>2</sub> 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<sub>2</sub> and 100 µM for PbCl<sub>2</sub> (59). Another study tested the adverse effects of CdCl<sub>2</sub>, MeHgCl<sub>2</sub> and PbCl<sub>2</sub> 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  $2.29 \times 10^{-3}$  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), where the effect of ambient fine particulate 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<sub>4</sub> 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  $\mu\text{M}$  of  $\text{CdCl}_2$  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  $\mu\text{M}$  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

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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.

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## Chapter 8: Appendices

### 8.1. Ethical clearance certificates



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)



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**

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)*

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Fakulteit Gesondheidswetenskappe  
Lefapha la Disaense tsa 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