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

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Abstract

Heavy metals are natural elements characterized by their relatively large atomic mass as well as a high density. It can be introduced into the ecosystem by the mining of heavy metals from deep within the earth's crust, thereby exposing the metals into air and water systems. Cigarette smoke is another source of heavy metal exposure and has been shown to have carcinogenic, toxic and genotoxic properties. Cadmium, lead and chromium are the most abundant metals found in cigarette smoke. In response to tobacco smoke exposure, endothelial cells release inflammatory and pro-atherogenic cytokines that are linked to endothelial dysfunction. Endothelial dysfunction, is directly related to the production of reactive oxygen species, leading to endothelial cell loss through necrosis and/or apoptosis. The current study aimed to investigate the effect of cadmium, lead and chromium, alone and as part of metal mixtures, on endothelial cells. The EA.hy926 endothelial cell line was exposed to different concentrations of each of these metals and their combinations and analysed using flow cytometric analyses with Annexin V. A clear trend was seen with the Pb + Cr as well as the triple combination group with the significant increase of early apoptotic cells. Scanning electron microscopy was used to study possible ultrastructural effects. Morphological changes observed with scanning electron microscopy included cell membrane damage and membrane blebbing at certain metal concentrations. In conclusion the exposure of endothelial cells to cadmium, lead, and

chromium, caused a disruption in cellular processes and morphology, possibly diminishing the protective ability of endothelial cells.

Keywords: Endothelial cells, Cadmium, Chromium, Lead, Scanning electron microscopy, Apoptosis, Necrosis

Introduction

The effect of heavy metal exposure on human health is an important area of interest due to their abundance in the environment. Increased abundance of heavy metals are a result of the use of a wide assortment of metals in industry and processes in daily life. [1] Heavy metals are considered dangerous to human health as they have the ability to bio accumulate in tissues, causing toxicity. [2]Tobacco smoke has carcinogenic, toxic, and genotoxic properties. Cigarette smoke is known to contain both inorganic and organic human carcinogenic compounds. 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).[2-4] Of these metals, the three most abundant metals are Cr, Cd and Pb.[3,5,6]

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 human body is unable to excrete Cd worsening the health effects of Cd exposure.[8] Cadmium-induced toxicity has been widely studied and has been shown to 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.[8,9]

Lead exhibits extreme toxicity even at very low exposure levels.[9,10] Lead, and its compounds are toxic and accumulate over a long period of time, as the body lacks excretory mechanisms for lead. This build-up of lead in the body is known as cumulative poisoning and will continue until a lethal quantity is reached. The toxicity of Pb compounds increases as their solubility increases.[10] Lead has no vital function in the human body and is therefore considered toxic as it is associated with adverse effects on the physiological functioning of the human body.[10]

Chromium is a trace element that the body needs in trace amounts and can exist in several oxidation states, however, the trivalent, Cr (III), and the hexavalent, Cr (VI), forms are the most found in the environment,[5,24] with Cr (VI) classified as a group 1 carcinogen mean that strong

linkages between the metal and cancer have been found. Consisting of mostly hydroxyl radicals, Cr (VI) can cause deoxyribose nucleic acid (DNA)-damage, by inducing single strand breaks leading to possible cell transforming effects. [5,24]

Although many studies have focused on 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,[11-17] 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.[17] In this study, the focus was on the effect of Cd, Cr and Pb alone and in combination on endothelial cells. In response to tobacco smoke exposure, endothelial cells release inflammatory and pro-atherogenic cytokines linked to endothelial dysfunction.[20] 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.[21] 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.[20]

In this study, the effects of Cd, Pb and Cr, alone and in combination on the endothelial cell line, EA.hy926 was tested by investigating the ability of these metals to induce apoptosis and necrosis and the possible morphological alterations associated with these cell death processes.

Materials and Methods

Metal Preparation

All metals used in this study, Cd, Pb and Cr were purchased from Sigma-Aldrich [St. Louis, Missouri(MO), United States of America (USA)], from which stock solutions were created and diluted using sterile ddH_2O.

Cell Culture

The EA.hy926 endothelial cell line was obtained from the Department of Physiology, Faculty of Health Sciences, University of Pretoria originally from the American Type Culture Collection (ATCC) and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin, streptomycin, amphotericin), (Sigma-Aldrich, St Louis, MO, USA) and incubated at 37°C in humidified

atmosphere with 5% carbon dioxide (CO_2). Once cells had reached 75 to 90% confluency, cells were trypsinised, counted and plated as required.

The concentrations of Cd, Pb and Cr and were obtained from the study conducted by Yaprak *et al*,[5] as indicated in Table 1 below (in µg/L).

Annexin V Assay

Cells were plated at a concentration of 7 x 10^4 cells/mL, in a 75cm^3 flask and left to attach overnight. Following overnight attachment, 1 mL of each metal sample at the X2 concentration (dissolved in medium) was added to each flask to make up a final volume of 10mL. The sample was left to incubate for 72 hours. Following the 72-hour incubation period, cells were trypsinized and centrifuged at 123 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. For analyses, 195 µL of cell suspension in binding buffer was placed in a micro-centrifuge tube along with 2.5 µL of Annexin V and 2.5 µ L of propidium iodide 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. A volume of 200 µL washed cells in binding buffer was then transferred to a Fluorescence Activated Cell Sorter (FACS) tube, vortexed and analysed using a Flow Cytometer (Beckman Coulter, Brea, California, USA) with the fluorescence of Annexin V (FITC) and PI was measured by logarithmic amplification. The number of events analysed for each gate/sample was 15 000. The flow cytometry results were analysed using Kaluza C software (Version 1.1.00003.20057 Beckman Coulter). Debris and doublets were gated out and excluded from further analyses.

Scanning Electron Microscopy

Cells were grown on glass cover slips in 24 well plates at a concentration of 5 x 10^4 cells/ mL, which have been coated with poly-L-lysine. The cells were then exposed to all three metals and concentration groups and incubated for 72 h. Following incubation, the cells were then fixed in 2.5% GA/FA in 0.075 M $NaPO_4$ buffer for 30 minutes. The cells were thereafter washed 3 times with 0.075 M $NaPO_4$ 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 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 hour, 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.

Statistical Considerations

All statistical analysis was carried out by using Graph Pad Prism analyzing software (version 8.4.3). A normality test using the Shapiro-Wilks and the D'Agostino- Pearson omnibus normality tests were performed to determine the normality of the data with a significance level (alpha) set to 0.05. Thereafter a One-Way ANOVA with Tukey's post hoc (parametric data) or a Kruskal Wallis test with Dunn's post hoc (non-parametric data) was performed to determine differences between controls and experimental samples. Statistical analysis was performed with a 95% confidence interval and a p-value of < 0.05 was considered significant.

Results

Flow Cytometry

All flow cytometry data is representative of at least three experimental repeats (n=3) ± standard error of mean (SEM) and presented as dot-plots, where viable cells are in the lower left quadrant [FITC (-) and PI (-)]; necrotic cells are in the lower right quadrant [FITC (-) and PI (+)]; early apoptotic cells are in the upper left quadrant [FITC (+) and PI (-)] and late apoptotic cells are in the upper right quadrant [FITC (+) and PI (+)]. The potential of heavy metals to induce apoptosis and necrosis was analysed using the Annexin V assay (Figure 1). A clear trend was seen with the Pb + Cr as well as the triple combination group with the significant increase of early apoptotic cells and a slight but non-significant increase in necrotic cells. A significant difference was seen between the Pb + Cr group and the triple combination group when compared to the medium control (Figure 2).

Scanning Electron Microscopy

The morphology of the EA.hy926 cells exposed to X2 metal concentrations was investigated using scanning electron microscopy and shown in Figures 3 - 10 below. Figure 3 is representative of the control samples where the varying normal morphology is shown. Some cells appeared elongated (Figure 3A) whereas others had a more rounded structure (Figure 3B and C). A high magnification micrograph of the cell membrane is shown in Figure 3D and

indicates a smooth surface with no perforations present. For each of the exposed groups, membrane damage in the form of perforations were observed as indicated by the white circles as well as the presence of membrane blebbing as indicated by the red arrows. Figures 4 - 6 are representative micrographs from the Cd, Pb and Cr- exposed groups respectively. In the Cd-exposed group (Figure 4), some membrane damage in the form of formation of perforations were observed as indicated by the white circles in B, D and F. The presence of membrane blebbing was also seen as indicated by the red arrows in Figure 4 C, D, E and F. In the Pb-exposed group (Figure 5), with perforations observed as indicated by the white circles in 6 B and F. Membrane blebbing was also seen as indicated by the red arrows in Figure 6 B, C, E and F. The Cr-exposed group (Figure 6) also showed membrane blebbing as indicated by the red arrows in Figure 6 A-F. Figures 7-9 are representative micrographs from the double combination exposure groups and indicate the Cd + Pb, Cd + Cr and Pb + Cr- exposed groups respectively. Figure 7 shows representative micrographs from the Cd + Pb-exposed group. In this group perforations were observed as indicated by the white circles in A, B and F, with Figure 7 F showing a distinctive tear in the membrane. Membrane blebbing was seen as indicated by the red arrows in Figure 7 A-F. Figure 8 shows representative micrographs from the Cd + Cr-exposed group. In this group some membrane damage indicated by perforations was observed as indicated by the white circles in B-F, with Figure 8F showing a distinctive tear in the membrane. Membrane blebbing was seen as indicated by the red arrows in Figure 8 A-F. Figure 9 shows representative micrographs of the Pb + Cr- exposed group that also presented with perforations as indicated by the white circles in Figure 9 B, C, and D with Figure 9 D showing excessive damage to the membrane. Membrane blebbing was present as indicated by the red arrows in Figure 9 A, B, E and F. Figure 10 shows micrographs from the Cd + Pb + Cr-exposed group. In this group some membrane damage were observed as indicated by the white circles in B and F, with Figure 10 B showing a hole in the membrane. The presence of membrane blebbing was seen as indicated by the red arrows in Figure 10 A-F.

In almost all 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 small tears or perforations was most evident in the Pb + Cr group. Membrane damage is a clear indication of necrosis and is consistent with the Annexin V assay (Figure 1) results. Table 2 summarizes the results obtained according to the prevalence of membrane blebbing and damage to the cell membrane as compared to the control.

Discussion

A study conducted by Kopp *et al*, [22] investigated the genotoxicity of 11 heavy metals, of which $CdCl_2$ and $PbCl_2$ was amongst the samples tested on hepatoma (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 $PbCl_2$[22]. 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).[23] These results were further substantiated by Lozi *et al*.[24] Cytotoxicity was seen at concentrations of 15 µM and above for each heavy metal. Based on the metal concentrations analysed (Table 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 lowest observed adverse effect level. This is a possible explanation for not observing extensive necrosis with flow cytometry and why no extensive membrane damage was present in the SEM micrographs.

The apoptotic and necrotic effects of the various metal concentrations alone and in combination were 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. An increase in the percentage necrosis was also seen in the Cd + Cr group, however this was not significant. A study conducted by Chukwuebuka *et al*.,[25] 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.[25]

A study conducted by Martínez-Nava *et al*.,[26] studied the effect of Cd on the concentration of essential metals in 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 12 h. 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.[26]

Analysis of the micrographs of the different metal groups obtained with scanning electron microscopy revealed the presence of membrane damage in the form of small tears or perforations 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 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*.,[27] to investigate differential acute lung cytotoxicity caused by heavy metals using a primary culture of alveolar type II cells. Results of both cytotoxicity and scanning electron microscopy analyses, indicated a dose-dependent relationship in each metal group tested (Cd, Pb, Hg and Ni) and significant differences in morphology were 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.[27] A study conducted by Sa *et al*.,[28] investigated the association between heavy metal exposure and cancer in renal cells. Using both scanning electron microscopy, 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.[28] Similar results were shown by Trabelisi *et al*.,[29] where the cytotoxic and genotoxic capabilities of Cd were 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 influence cell cycle events. Mitochondria alterations were seen with TEM analysis, which substantiated the results obtained from the MTT assay to determine cytotoxicity. It can therefore be assumed that the toxicity of heavy metals is not necessarily linked to DNA mutations, but perhaps rather to the inflammation associated with damage to the endothelial layer (as indicated by the findings of this study) which is present continuously and that can lead to adverse cardiovascular and respiratory conditions in addition to renal- and hepatic damage.

Conclusion

Exposure of endothelial cells (EA.hy926) to Cd, Pb and Cr alone and in combination at three different concentrations (X0.1, X1 and X2) showed apoptosis and a slight, but not significant increase in necrosis at increased concentrations of the Pb + Cr and triple combination groups with flow cytometry analysis. Morphological changes were observed with SEM, with almost all the X2 concentrations of metals showing either damage to the cell membrane, the presence

of cell blebs, 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. The current study revealed changes on ultrastructural level, even at very low metal concentrations, that should be the basis for further investigation.

References

1. Mahurpawar M. Effects of heavy metals on human health. *Int J Res Granthaalayah.* 2015. 530:1-7
2. Bernhard D, Rossmann A, Wick G. Metals in cigarette smoke. *IUBMB life*. 2005. 57(12):805-9.
3. Ashraf MW. Levels of Heavy Metals in Popular Cigarette Brands and Exposure to These Metals via Smoking. *Sci World J*. 2012. 2012:729430.
4. Engida AM, Chandravanshi BS. Assessment of heavy metals in tobacco of cigarettes commonly sold in Ethiopia. *Chem Int*. 2017. 3(3):212-8.
5. Yaprak E, Yolcubal İ. Presence of toxic heavy metals in platelet-rich fibrin: a pilot study. *Biol Trace Elem Res*. 2019. 191(2):363-9.
6. Massadeh A, Gharibeh A, Omari K, Al-Momani I, Alomari A, Tumah H, et al. Simultaneous determination of Cd, Pb, Cu, Zn, and Se in human blood of Jordanian smokers by ICP-OES. *Biol Trace Elem Res*. 2010. 133(1):1-11.
7. Khan AR, Awan FR. Metals in the pathogenesis of type 2 diabetes. *J Diabetes Metab Disord*. 2014. 13(1):1-6.
8. Tang L, Su J, Liang P. Modeling cadmium-induced endothelial toxicity using human pluripotent stem cell-derived endothelial cells. *Sci Rep*. 2017. 7(1):1-12.
9. Patra RC, Rautray AK, Swarup D. Oxidative stress in lead and cadmium toxicity and its amelioration. *Vet Med Int*. 2011. 2011:457327.
10. Attar T. A mini-review on importance and role of trace elements in the human organism. *Chem Rev Lett*. 2020. 3(3):117-30.
11. van Rensburg MJ, van Rooy M, Bester MJ, Serem JC, Venter C, Oberholzer HM. Oxidative and haemostatic effects of copper, manganese and mercury, alone and in combination at physiologically relevant levels: An ex vivo study. *Hum Exp Toxicol*. 2019. 38(4):419-33.

12. Venter C, Oberholzer HM, Bester J, van Rooy M-J, Bester MJ. Ultrastructural, Confocal and Viscoelastic Characteristics of Whole Blood and Plasma After Exposure to Cadmium and Chromium Alone and in Combination: An Ex Vivo Study. *Cell Physiol Biochem*. 2017. 43:1288-300.

13. Markiewicz-Górka I, Januszewska L, Michalak A, Prokopowicz A, Januszewska E, Pawlas N, et al. Effects of chronic exposure to lead, cadmium, and manganese mixtures on oxidative stress in rat liver and heart. *Arh Hig Rada Toksikol*. 2015. 66(1):51-62.

14. Li C, Zhou H-M. The role of manganese superoxide dismutase in inflammation defense. *Enzyme Res.* 2011. 2011:387176-.

15. Arbi S, Oberholzer HM, van Rooy MJ, Venter C, Bester MJ. Effects of chronic exposure to mercury and cadmium alone and in combination on the coagulation system of Sprague-Dawley rats. *Ultrastruct Pathol*. 2017. 41(4):275-83.

16. Naidoo SVK, Bester MJ, Arbi S, Venter C, Dhanraj P, Oberholzer HM. Oral exposure to cadmium and mercury alone and in combination causes damage to the lung tissue of Sprague-Dawley rats. *Environ Toxicol Pharmacol*. 2019. 69:86–94.

17. Venter C, Oberholzer HM, Taute H, Cummings FR, Bester MJ. An in ovo investigation into the hepatotoxicity of cadmium and chromium evaluated with light-and transmission electron microscopy and electron energy-loss spectroscopy. *J Environ Sci Health A Tox Hazard Subst Environ Eng*. 2015. 50(8):830-8.

18. Onat D, Brillon D, Colombo PC, Schmidt AM. Human vascular endothelial cells: a model system for studying vascular inflammation in diabetes and atherosclerosis. *Curr Diab Rep*. 2011. 11(3):193-202.

19. Wang M, Hao H, Leeper NJ, Zhu L. Thrombotic regulation from the endothelial cell perspectives. *Arterioscler Thromb Vasc Biol*. 2018. 38(6):e90-e5.

20. Messner B, Bernhard D. Smoking and cardiovascular disease: mechanisms of endothelial dysfunction and early atherogenesis. *Arterioscler Thromb Vasc Biol*. 2014. 34(3):509-15.

21. Healthcare S. Endothelial Dysfunction Stanford Healthcare [Available from: https://stanfordhealthcare.org/medical-conditions/blood-heart-circulation/endothelial-dysfunction.html#:~:text=Endothelial%20dysfunction%20is%20a%20type,and%20causes%20chronic%20chest%20pain.

22. Kopp B, Zalko D, Audebert M. Genotoxicity of 11 heavy metals detected as food contaminants in two human cell lines. *Environ Mol Mutagen*. 2018. 59(3):202-10.

23. Abdelsalam EEE, Banďouchová H, Heger T, Kaňová M, Kobelková K, Němcová M, et al. Reproductive toxicity of heavy metals in fallow deer in vitro. *Acta Veterinaria Brno*. 2021. 90(3):277-86.

24. Lozi AA, Dias FCR, Silva J, Araujo DC, Gonçalves IC, Melo FCSA, et al. Comparative Toxicology of Heavy Metals Arsenate, Arsenite, Cadmium, Chrome Vi, Lead, and Nickel in the Testes of Adult Swiss Mice After Acute Exposure. 2022. doi.org/10.21203/rs.3.rs-1949599/v1

25. Chukwuebuka TE, Francesco M, Adebayo AO. In vitro cyto-toxic assessment of heavy metals and their binary mixtures on mast cell-like, rat basophilic leukemia (RBL-2H3) cells. *Chemosphere*. 2019. 223: 686-693

26. Martínez-Nava GA, Mendoza-Soto L, Fernández-Torres J, Zamudio-Cuevas Y, Reyes-Hinojosa D, Plata-Rodríguez R, et al. Effect of cadmium on the concentration of essential metals in a human chondrocyte micromass culture. *J Trace Elem Med Biol*. 2020. 62:126614.

27. Takano Y, Taguchi T, Suzuki I, Balis JU, Yuri K. Cytotoxicity of Heavy Metals on Primary Cultured Alveolar Type II Cells. *Environment Res*. 2002. 89(2):138-45.

28. Sá I, Semedo M, Cunha ME. Kidney cancer. Heavy metals as a risk factor. *Porto Biomed J*. 2016. 1(1):25-8.

29. Trabelsi F, Khlifi R, Goux D, Guillamin M, Hamza-Chaffai A, Sichel F. Genotoxic effects of cadmium in human head and neck cell line SQ20B. *Environ Sci Pollut Res Int*. 2016. 23(16):16127-36.

Table 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* (5).

Table 2 - 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 (+++). Cd: cadmium; Pb: lead; Cr: chromium.

Figure 1

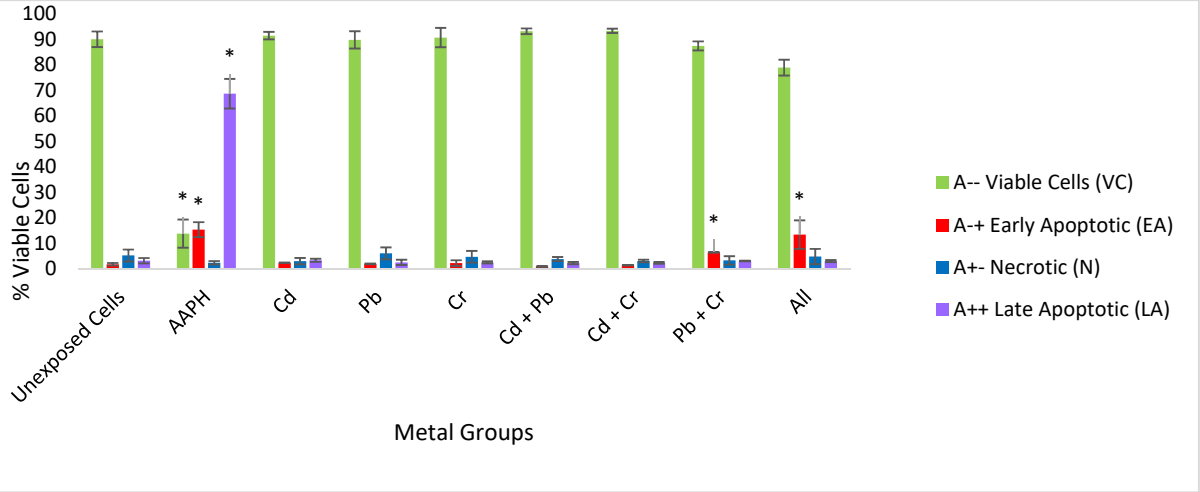
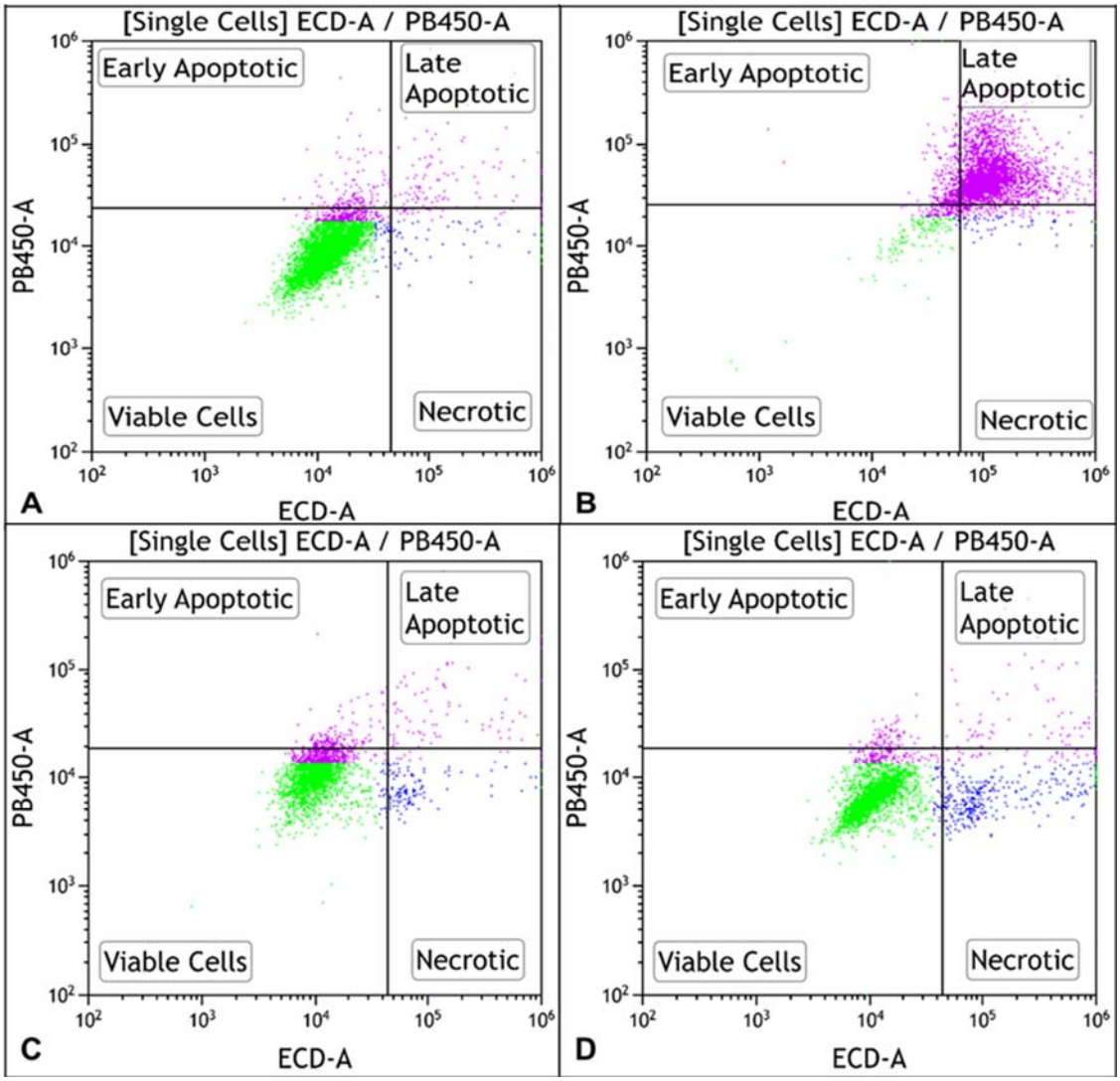

100
90
80
70
60
50
40
30
20
10
0
% Viable Cells
Unexposed Cells
AAPH
Cd
Pb
Cr
Cd + Pb
Cd + Cr
Pb + Cr
All
Metal Groups
A-- Viable Cells (VC)
A-+ Early Apoptotic (EA)
A+- Necrotic (N)
A++ Late Apoptotic (LA)

Figure 2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Late Apoptotic
Viable Cells
Necrotic
PB450-A
ECD-A
A
B
C
D

Figure 3

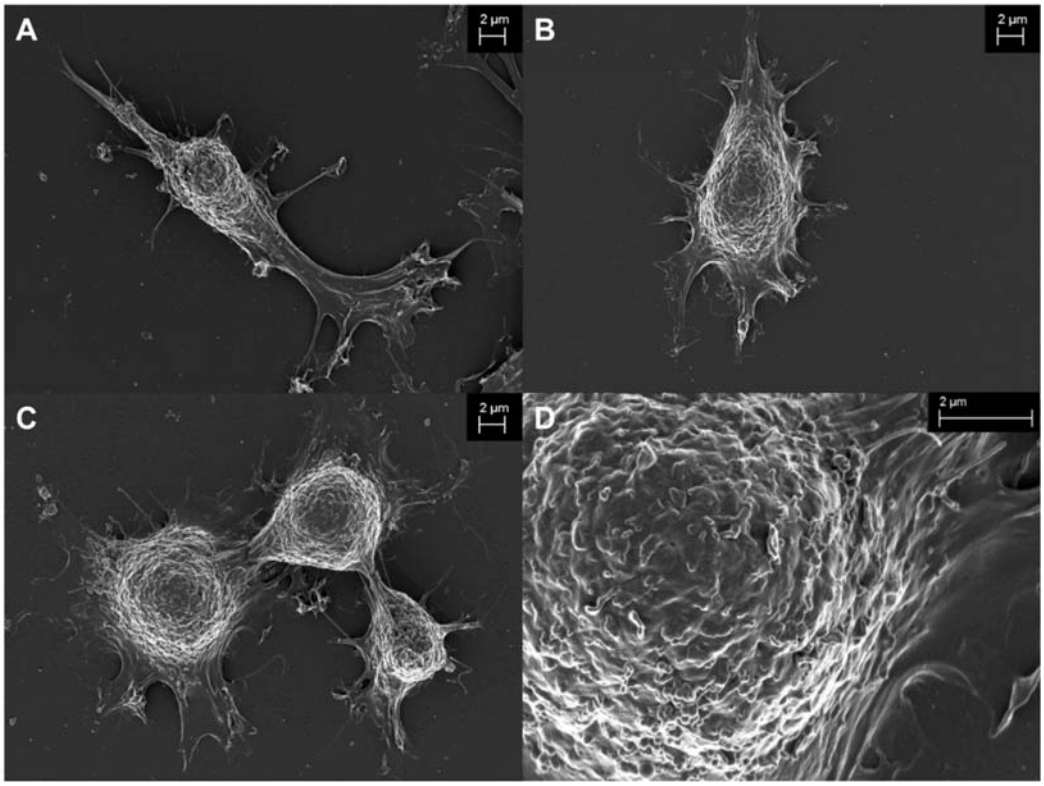

Figure 4

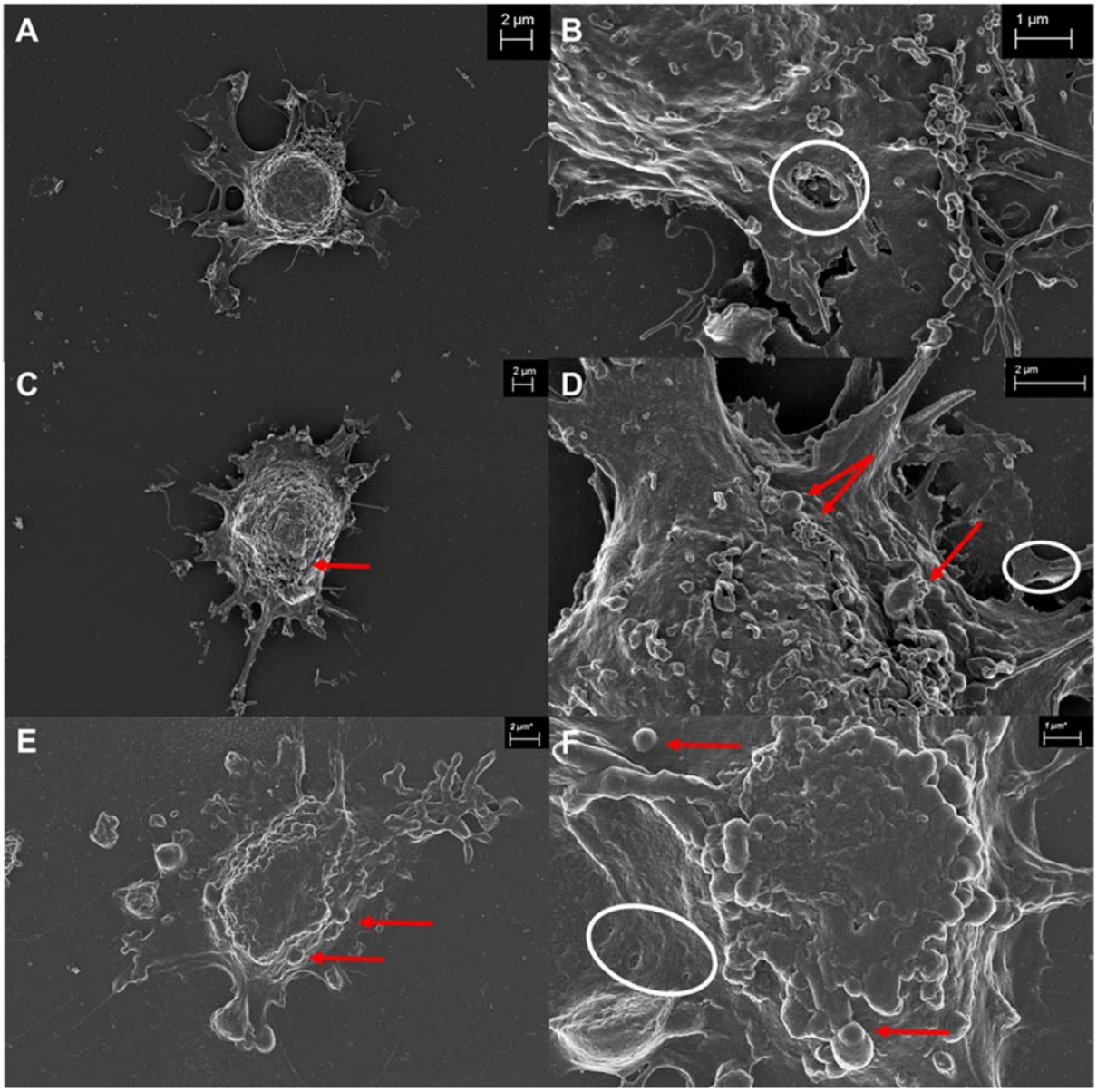

Figure 5

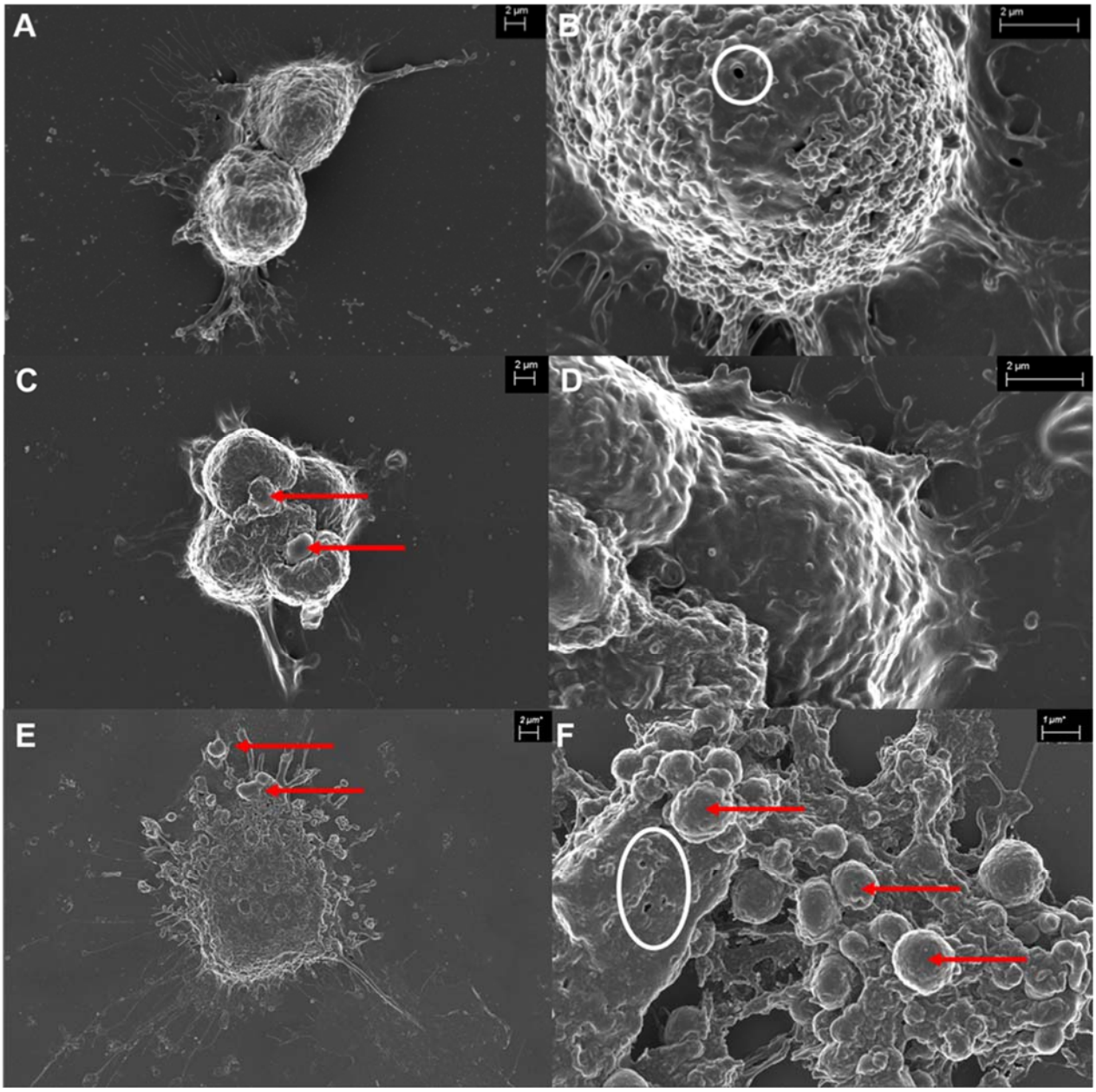

Figure 6

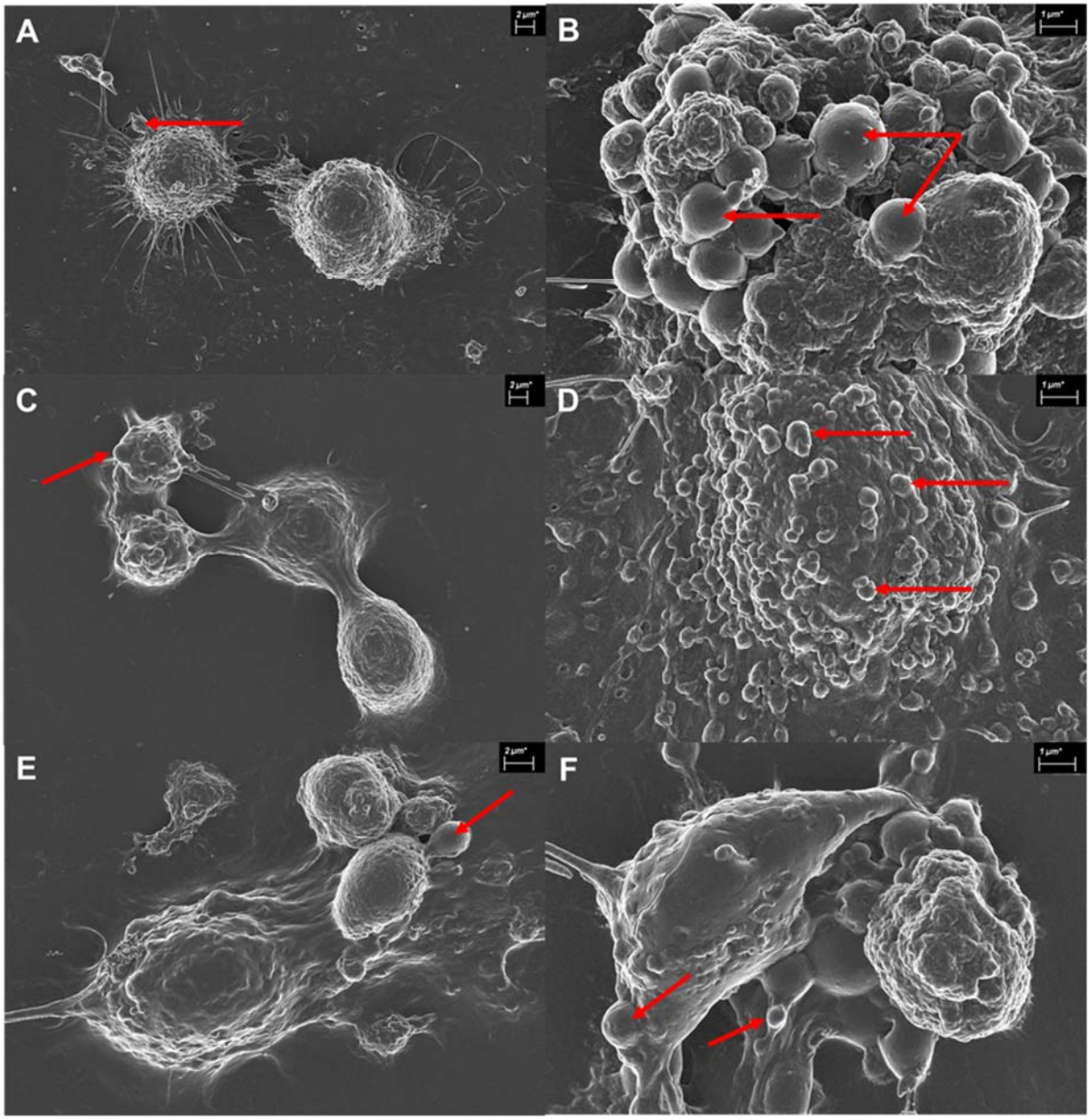

Figure 7

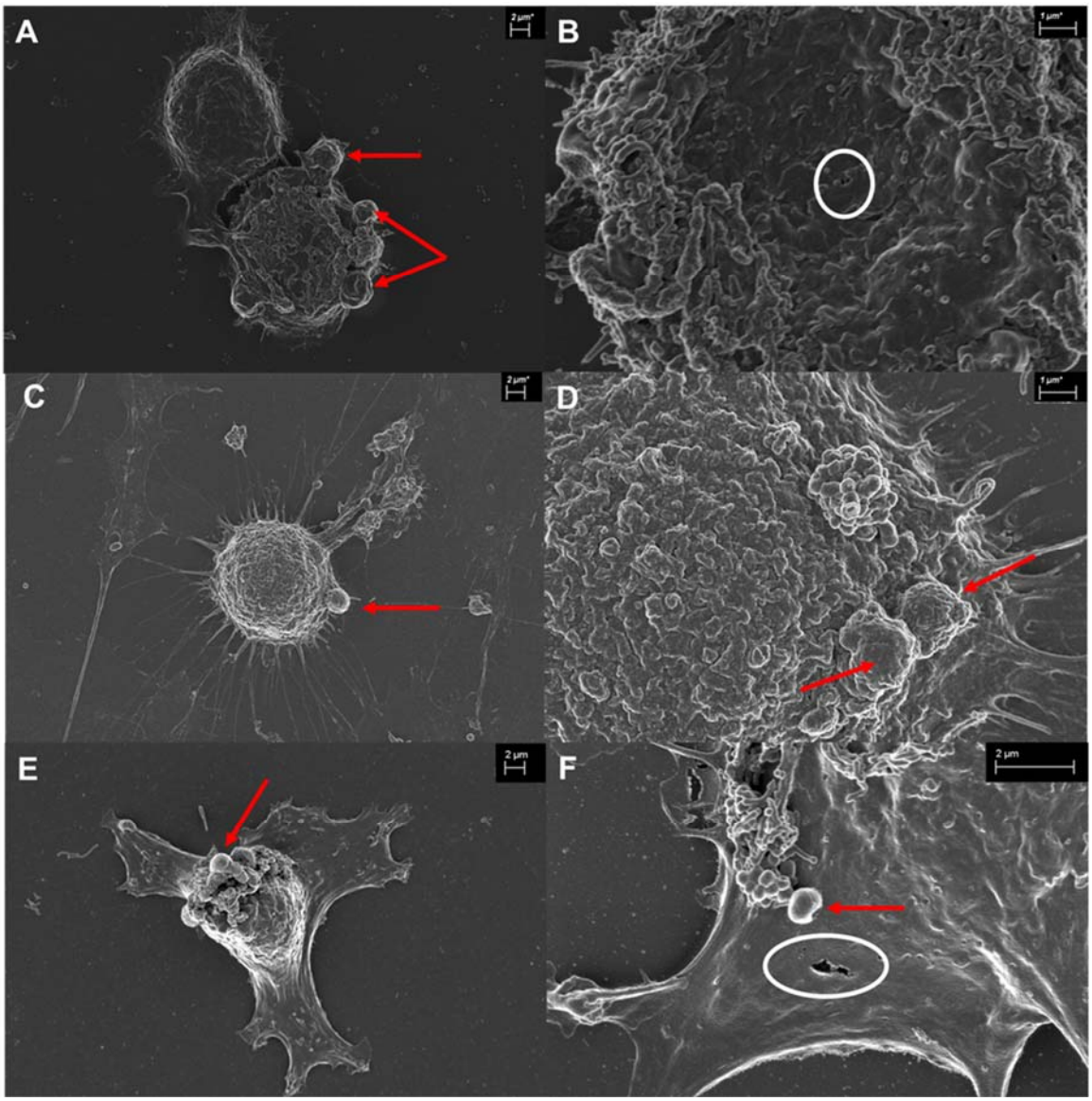

Figure 8

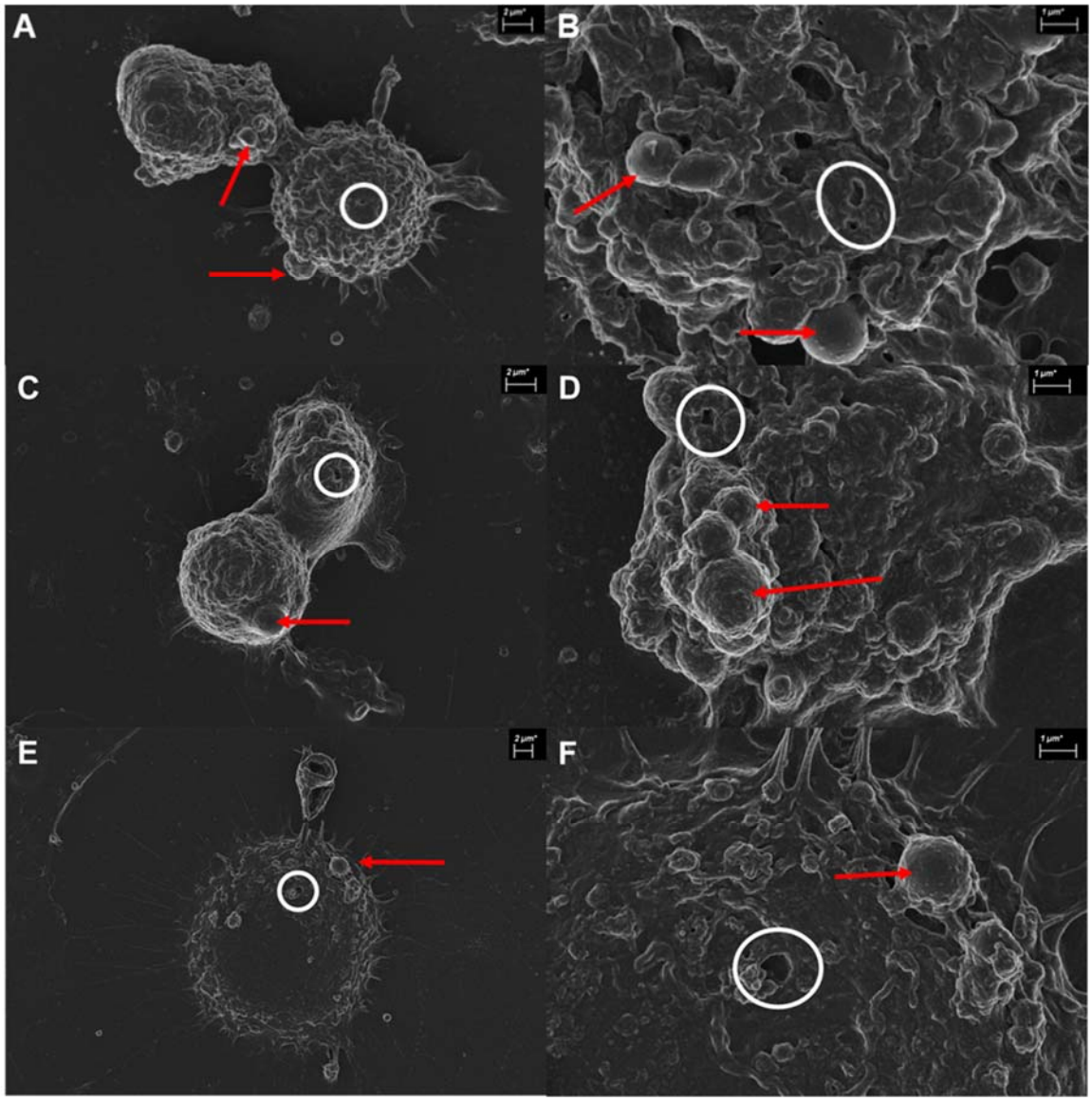

Figure 9

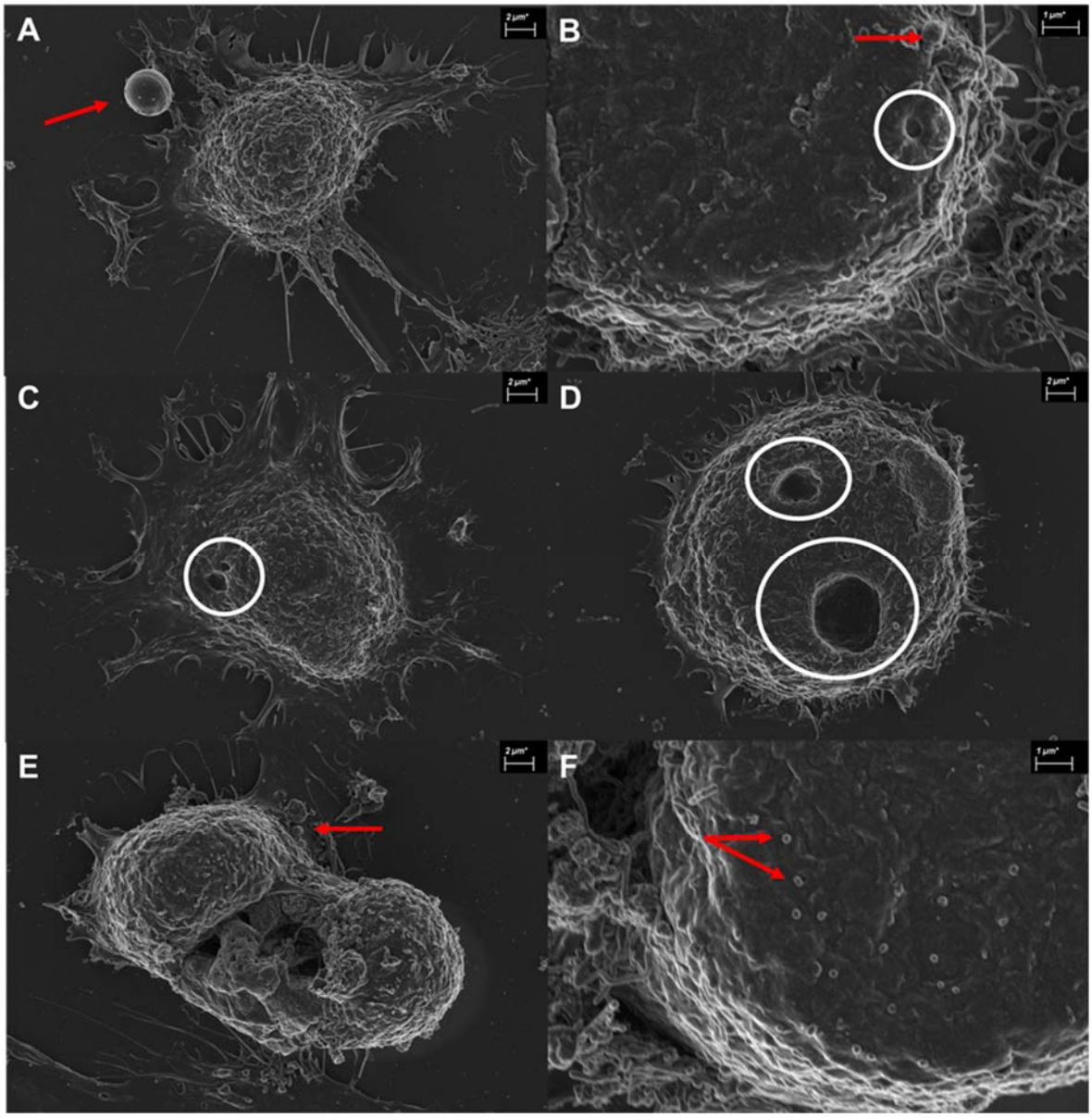

Figure 10

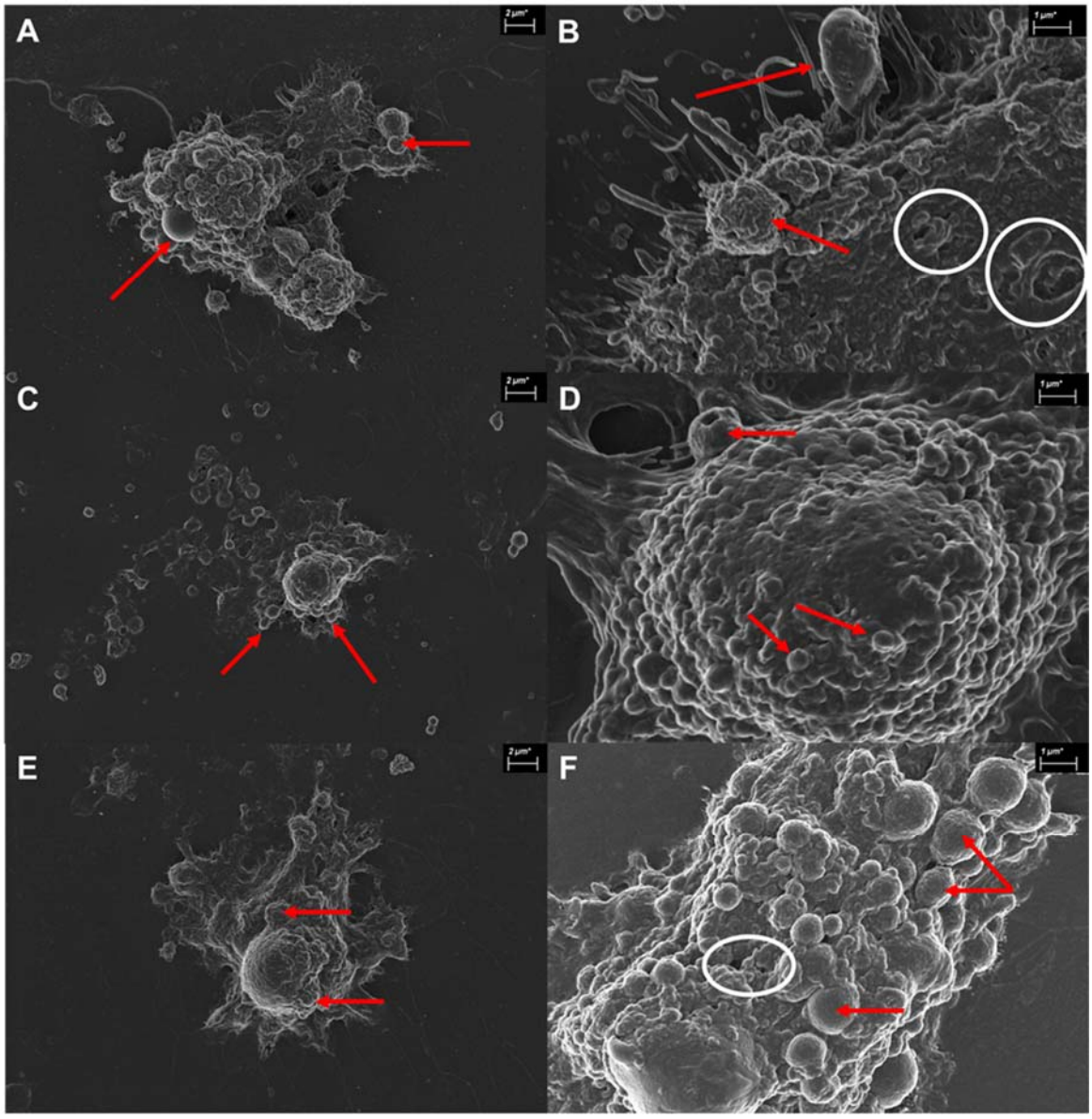

Figure 1 - Graph indicating the Annexin V results of the X2 concentrations of metals using flow cytometry. VC- viable cells which stained negative for both Annexin V and PI, EA- Early Apoptotic, stained positive for Annexin V and negative for PI, N- necrotic, stained negative for Annexin V and positive for PI and LA- Late Apoptotic, stained positive for both Annexin V and PI. Results are expressed as mean ± SEM of three independent triplicate experiments with p-value of <0.05. * - Significance was seen when compared to the control cells

Figure 2 - PI versus Annexin V dot-plots. Significance was seen between the Pb + Cr sample and the triple combination sample when compared to the medium control. A- Medium control, B- AAPH (1 mg/mL) positive control, C- Pb + Cr Group and D - All Group

Figure 3 - 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)

Figure 4 - 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

Figure 5- 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

Figure 6- 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.

Figure 7 - 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.

Figure 8 - 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

Figure 9 - 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.

Figure 10 - 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.