Whole blood ultrastructural alterations by mercury, nickel and manganese alone and in combination: An *ex vivo* investigation

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Abstract

The distribution of metals across the environment is increasingly becoming a major concern as they not only pollute the environment but also pose a danger to humans and animals. Human exposure to heavy metals often occurs as a combination of metals the synergistic effects of which can be more toxic than a single metal. The aim of this study was to investigate the effects that the metals mercury (Hg), nickel (Ni) and manganese (Mn) alone and in combination have on erythrocyte morphology and other components of the coagulation system using the haemolysis assay, scanning electron microscopy (SEM), and confocal laser scanning microscopy. Human blood was exposed to the heavy metals *ex vivo*, and percentage haemolysis was determined. Ultrastructural analysis of erythrocytes, platelets and fibrin networks was performed using SEM. Analysis of phosphatidylserine (PS) flip-flop was determined using confocal laser scanning microscopy. At the highest concentration of 10,000× the World Health Organization safety limit, all the metals caused haemolysis. The results showed that the exposure of erythrocytes to Hg alone and in combination with other metals displayed more haemolysis compared to Ni and Mn alone and in combination. Components of the coagulation system showed ultrastructural changes, including the formation of echinocytes and the activation of platelets with all single metals as well as the combinations. Confocal laser scanning microscopy analysis showed the presence of PS on the outer surface of the echinocytes that were exposed to metals alone and in combination. It can, therefore, be concluded that these heavy metals have a negative impact on erythrocytes and the coagulation system.

Key words: Mercury, Nickel, Manganese, Erythrocytes, Platelets, Fibrin fibers

Introduction

Erythrocytes and the components of the coagulation system functions to prevent the loss of blood from a damaged blood vessel. Platelets and fibrin networks form a stable thrombus that prevents excessive bleeding [1]. Erythrocytes are highly susceptible to changes in the environment evident in alterations seen in erythrocyte membranes when exposed to various endogenous and exogenous factors [2]. Endogenous and exogenous sources of reactive oxidative species (ROS) interact with erythrocytes, which may cause damage to erythrocytes visualised in changes in the morphology of erythrocytes and a reduction in the functioning of the erythrocytes [3]. Erythrocytes play a vital role in the transportation of oxygen molecules in the body. Alterations in the

morphological changes may weaken its oxygen carrying capacity and reduce its functioning [4]. The widespread usage of heavy metals within different sectors such as the agricultural, domestic and industrial areas raises an alarming concern over their impact on the environment and human health. Although some metals are known to pose a danger to human health, others such as copper (Cu) and chromium (Cr) are considered essential metals to humans and animals [5]. Chronic exposure to heavy metals through food and water consumption and the polluted air as a result of agricultural, cigarette smoking, domestic and industrial areas, however, may result in various ailments such as cardiovascular diseases, neurological and behavioural disorders, and those affecting the immune system and kidneys [6-8]. In addition, external factors such as metals can therefore impede the effective functioning of erythrocytes, platelets and fibrin networks. The World Health Organization (WHO) has defined a normal range for the consumption of heavy metals for the general population. This was done to safeguard the population from deficiency and toxicity of metals [9-11] known to be a major concern in the health sector [9]. Mercury (Hg), nickel (Ni) and manganese (Mn) used in this study where chosen based on the likelihood of humans being exposed to these metals in South Africa. In this study, the effects of the metals Hg, Ni and Mn, alone and in combination were investigated on the coagulation system by using the haemolysis assay, scanning electron microscopy and confocal laser scanning microscopy.

Materials and Methods

Ex vivo model

Five millilitres of venous, human blood was collected in a vaccutainer containing 3.2% sodium citrate from ten healthy, male, donors by a trained phlebotomist after written informed consent was obtained. Each tube was labelled with the sample number and the date on which the blood was collected so as to maintain the donor's anonymity. Ethical approval was obtained from the Research Ethics Committee of the Faculty of Health Sciences for the use of human blood (12/2018).

Haemolysis assay

Whole blood was centrifuged at 3000 xg for 10 minutes, after which the plasma (containing platelets) and buffy coat (containing leukocytes) was removed. The erythrocytes were washed twice with isoPBS (0.137 M NaCl, 3 mM KCl, 1.9 mM NaH_2PO_4, 8.1 mM Na_2HPO_4, pH 7.4), by re-suspending the erythrocytes in isoPBS and centrifuging between washes, at 1400 xg for 3 minutes. A 5% (v/v) blood suspension was made by diluting the packed erythrocytes in isoPBS.

A positive control, 2% sodium dodecyl sulphate (SDS) solution was used to induce 100% haemolysis and a negative control (isoPBS) was used to represent 0% haemolysis. The 5% blood suspension was then exposed to the metals, alone and in combination. The concentration ranges of the various metals included: 1x, 10x, 100x, 1000x and 10000x the WHO safety level standards for each respective metal. These concentrations were included as they represent the low and high possible exposure of heavy metals to humans. All the exposed samples were incubated for 16 hours at 37°C. The samples were centrifuged at 1400 xg for 2 minutes after which equal volumes of the supernatant was transferred to a 96-well plate and the absorbance was read at 570 nm. The results were expressed as percentage haemolysis, using the formula % Haemolysis = (A_{sample} - $A_{0\%}$ / $A_{100\%}$ - $A_{0\%}$) X 100. 0% was isoPBS and 100% was SDS.

Scanning electron microscopy (SEM)

The effect of the metals, alone and in combination, on the morphological changes to erythrocyte membranes together with platelets and fibrin networks were investigated using SEM. Whole blood was exposed to the different metals, alone and in combination and then incubated for 10 minutes at room temperature. This exposure time was determined to be the optimal period for exposure through a time-based study comparing samples exposed for 10 minutes, 30 minutes and 16 hours. Blood smears were made on round glass cover slips, with and without the addition of 10µL human thrombin (20 U/mL). The cover slips were dried for 10 minutes and washed in phosphate buffered saline (PBS) (0.075 M Na_2HPO_4, 0.2 M $NaH_2PO_4.H_2O$, and 0.2 M NaCl), for 20 minutes. The samples were then fixed in a 2.5% glutaraldehyde/formaldehyde (GA/FA) solution in 0.075M PBS for 30 minutes and then washed three times in PBS. A secondary fixation step was performed in 1% osmium tetroxide for 30 minutes and washed again as explained in the previous step. The samples were dehydrated using serial dehydration of 30%, 50%, 70% and 90% ethanol (EtOH), followed by three changes of absolute EtOH. Absolute EtOH was replaced with hexamethyldisilazane (HMDS) and allowed to air dry. The dried samples were mounted on aluminium stubs, coated with carbon and viewed with an Ultra Plus FEG SEM (Zeiss, Oberkochen, Germany).

Confocal laser scanning microscopy

Control blood was exposed to isoPBS (negative control) and Mellitin (an apoptosis inducing agent) that was used as positive control [12]. One mL of the blood was centrifuged at 3000 xg for ten minutes at room temperature to isolate the erythrocytes. The supernatant was discarded (containing plasma, leukocytes and platelets) and the remaining erythrocyte pellet was washed

twice with a 0.075 M PBS (pH 7.4) for 3 minutes, where after it was washed once with Annexin V binding buffer for 3 minutes. A volume of 5 uL of the Annexin V probe was added to the blood and incubated for 90 minutes at room temperature in the dark. After incubation, the samples were washed twice with 0.075 M PBS (pH 7.4) and once with Annexin V binding buffer for 3 minutes each, to remove unbound antibodies. A volume of 10µL of the prepared sample was mounted on a glass slide and covered with a coverslip. The samples were viewed with the Zeiss LSM 880 confocal laser scanning microscope with Airyscan (Carl Zeiss Microsocpy, Oberkochen, Germany).

To visualize the erythrocytes, two different lasers were used with different filters and beam splitters after which the respective images were overlaid to show which RBCs have a PS flip present on the membrane. To view the auto-fluorescence of all the cells present on the slide, the 405 nm laser was used to excite naturally occurring fluorescence found in RBCs, and a red colour was assigned to this fluorescent signal. In an unstained sample, the 405 lasers were used together with the 465 nm-505 nm Band pass (BP) and 525 nm long pass (LP) filters and the 488/405 nm beam splitters have auto-fluorescence and may be used as a contrasting method against the Annexin-V binding, in the case where PS flip is present. To visualize Annexin-V binding the 488nm laser was used with the 495-550nm BP filters and the 488/405nm beam splitters, and showed a green fluorescence, indicating the presence of a PS on the erythrocyte's membranes.

Statistical analysis

Of primary interest in this study was to compare three heavy metals, alone and in combination, at five different concentrations using the haemolysis assay. A biostatistician was consulted to determine the suitable statistical tests (see Appendix 8.3). By convention, the sample size when comparing the heavy metal combinations in an analysis of variance (ANOVA), for a two-factor study design (heavy metals combination × concentration), the aim is to have at least 30 degrees of freedom for the error term (residuals). In this study, from three experiments, there was 70 degrees of freedom where we considered treatment at seven levels (heavy metal combinations), concentration at five levels along with an interaction term for treatment by consideration. Data followed Gaussian distribution and since the experiments were done under well-controlled conditions, both analytical and biological variation was expected to be low. Data summary was within treatment combination (heavy metal combination × concentration) for one assay. Data analysis employed an appropriate ANOVA for this two-factor study design with an interaction term. Stata Release 14 statistical software was employed and post hoc testing used the very

flexible margins command in Stata. Testing was done at the 0.05 level of significance and the family-wise type I error was included.

Results

Haemolysis assay

The haemolysis assay was used to detect the haemoglobin content released from the erythrocytes as an indication of the effect of the heavy metals on erythrocyte membrane integrity. The metals used in the study alone and in double and triple combinations resulted in varying degrees of haemolysis at different concentrations between x1 - x10000 the WHO safety limit. The Hg (x1) with p-value of <0.9731 and Hg+Ni+Mn (x1) with p-value of <0.9969 induced haemolysis although it is statistically insignificant compared to isoPBS. Ni+Mn (x10) showed minimal haemolysis and was statistically insignificant with a p-value 0.9364. Ni(x100) and Mn+Ni(x100) caused haemolysis. Mn(x1000) and Ni+Mn (x1000) induced haemolysis. Haemolysis did not occur in the following metals (x1) Ni, Mn, Hg+Ni, Hg+Ni, Mn+Ni and (x10) Hg, Ni, Mn, Hg+Ni, Hg+Mn and Hg+Ni+Mn and (x100) Hg, Mn, Hg+Ni, Hg+Mn Hg+Ni+Mn and (x1000) Hg, Ni, Hg+Ni, Hg+Mn and Hg+Ni+Mn. Figure 1A represents the x10 000 concentration all the groups that induced haemolysis. The percentage haemolysis for all groups was well above 30% except Ni which exhibited the lowest percentage haemolysis (Hg 68.1%, Ni 1.6%, Mn 30.4%, Hg+Ni 66.7%, Hg+Mn 33.4%, Ni+Mn 45.6% and Hg+Ni+Mn 58.9%). Hg, Hg+Ni, Hg+Mn, Mn+Ni and Hg+Ni+Mn were all significantly different to the negative control (isoPBS). Figure 1B shows a comparison of all the metals groups with the varying concentration ranges.

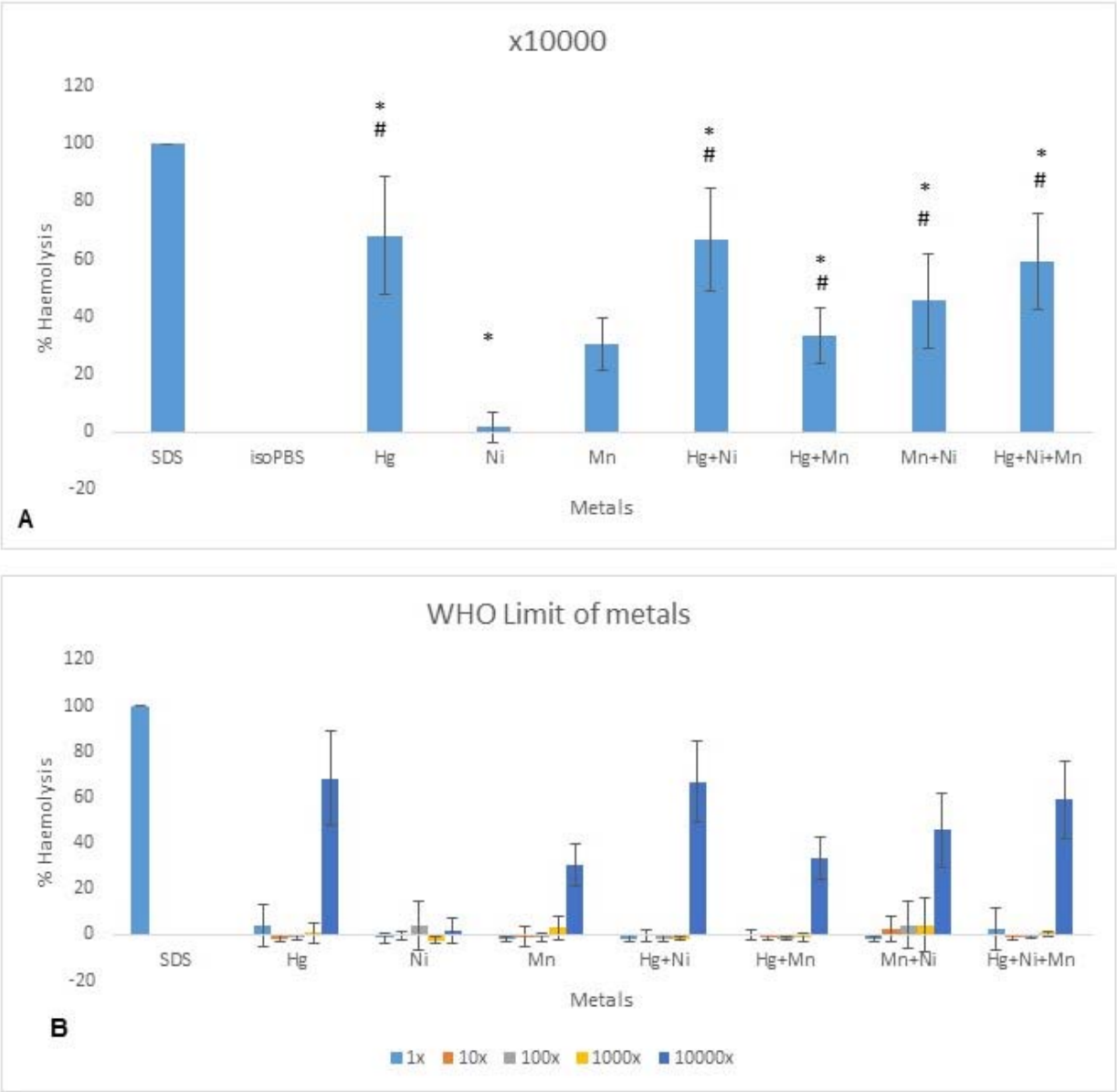

Figure 1: A comparison of heavy metal induced percentage haemolysis. The haemolytic effects of Hg, Ni and Mn at A) X1, B) X10, C) X100, D) X1000, E) X 10000 and F) overall representation of the haemolysis of all the metals the WHO safety level standards of each metal. The data is an average of 10 independent experiments expressed as the mean ± standard error of mean (SEM) compared to positive control (SDS) 100% haemolysis and negative control (NC) (isoPBS0) 0% haemolysis. # indicates significance compared to the NC and * indicates significance compared to other metal groups.

The haemolysis assay only provided the effect of the metal combination on the erythrocyte's membrane, resulting in the appearance of free hemoglobin in the plasma. The assay did not propose any information on the interaction between the double and triple combinations of metals

containing Hg, Ni and Mn across the five point increasing concentrations. There are three types of interactions found amongst metal combinations. An antagonistic effect is when two or more metals produce contrasting effects. For example, when Hg has an increasing effect on the percentage haemolysis but Mn has a decreasing effect on haemolysis. An additive effect is when the combination of the metals produces the same percentage haemolysis compared to the single metal effect. A synergistic effect is when the combination of metals produces a greater haemolysis percentage compared to the individual metal. Analysis of the combined effects was achieved using the model deviation ratio (MDR) method of $MDR=O_v/E_v$. An additive effect is indicated where $0.5<MDR<2$, antagonism where $MDR<0.5$ and synergism where $MDR>2$ [13-14]. Table 1 below indicates the interaction observed on the combined metals.

Table 1: Effect of metal combinations on haemolysis results

Effects	Metals
Synergistic	Hg+Ni (x10, x10 000) Hg+Mn (x10) Ni+Mn (x10, x100, x1000, x10 000) Hg+Ni+Mn (x10 000)
Antagonistic	Hg+Ni (x1, x100, x1000) Hg+Mn (x1, x100, x1000, x10 000) Ni+Mn (x1) Hg+Ni+Mn (x1, x10, x100,)
Additive	Hg+Ni+Mn (x10 000)

Scanning electron microscopy

The effects of Hg, Ni and Mn alone and in combination on the morphology of erythrocytes were studied using SEM. The x1 WHO safety limit concentration was used. The images are representative of erythrocytes acquired from blood smears with and without the addition of thrombin. The addition of thrombin induces fibrin formation through the conversion of fibrinogen to fibrin [15]. Figure 2 shows the scanning electron micrographs of erythrocytes exposed to metal concentrations at x1 the WHO limit. Healthy erythrocytes are shown in Figure 2A, with a typical biconcave structure. In Figure 2B, Hg exposure induced the presence of spike-like nodules (indicative of an echinocytes). Nickel exposure (Figure 2C) caused cell blebbing, which was also seen with Mn exposure (Figure 2D). Echinocytes were observed in the Hg+Ni and Hg+Mn exposed groups (Figure 2E and 2F). Pinching of the erythrocyte membrane was seen in the Mn+Ni group (Figure 2G) and Hg+Ni+Mn exposure caused a loss of biconcave morphology in the erythrocytes as shown in Figure 2H.

Figure 3 shows the scanning electron micrographs of platelets exposed to metal concentrations at x1 the WHO limit. Figure 3A shows normal morphology of platelets with presence of

pseudopodia. Contact activation is expected during the preparation of the samples with some pseudopodia formation, as seen in the figure representing the healthy platelets. Figure 3B to G shows activated platelets with presence of pseudopodia to a greater degree than that caused by possible contact activation as seen in the control sample in Figure 3A. In Figure 3H the presence of pseudopodia and membrane spreading can be seen.

Figure 4 shows scanning electron micrographs of whole blood with thrombin exposed to heavy metal concentration at x1 the WHO limit. Figure 4A represents the fibrin network of a control sample. Healthy fibrin clots will also contain thin, thick and taut fibres. In Figure 4B and 4C thick, thin and less taut fibres compared to the control and the presence of echinocytes was observed. Thick and less taut fibres are seen in the Mn exposure, thin fibres are seen in the Hg+Ni and Ni+Mn exposed groups and Hg+Mn exposed group showed thick and thin fibres.

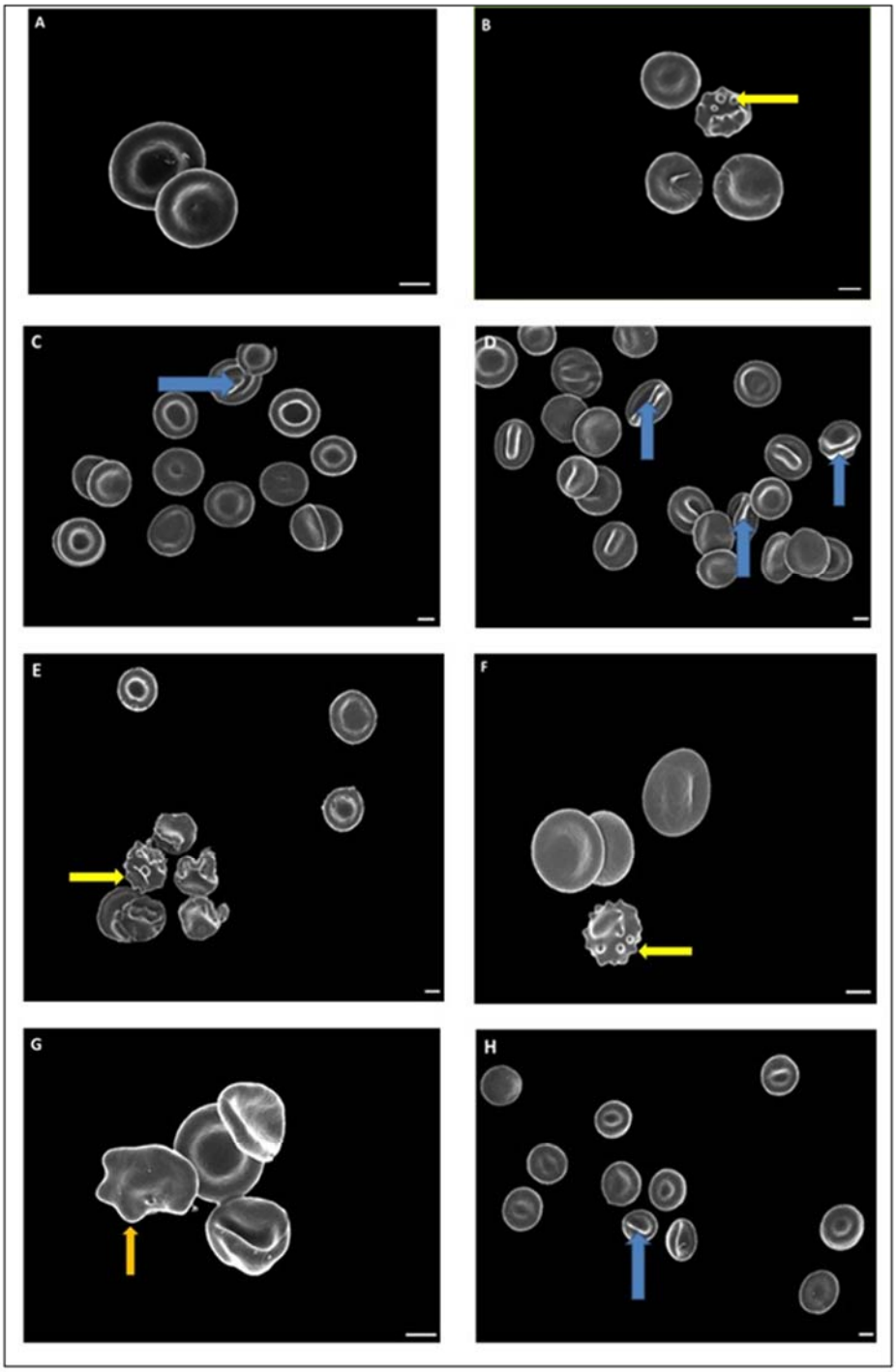

Figure 2: Scanning electron micrographs of whole blood without thrombin exposed to Hg, Ni and Mn, alone and in combination at x1 the WHO safety limit. (A): Control with normal biconcave erythrocytes. (B): Hg, (C): Ni, (D): Mn, (E): Hg+Ni, (F): Hg+Mn, (G): Ni+Mn, (H): Hg+Ni+Mn. Scale bars=2μm. Blue arrows bulging appearance, Yellow arrows echinocytes (nodule like spikes).

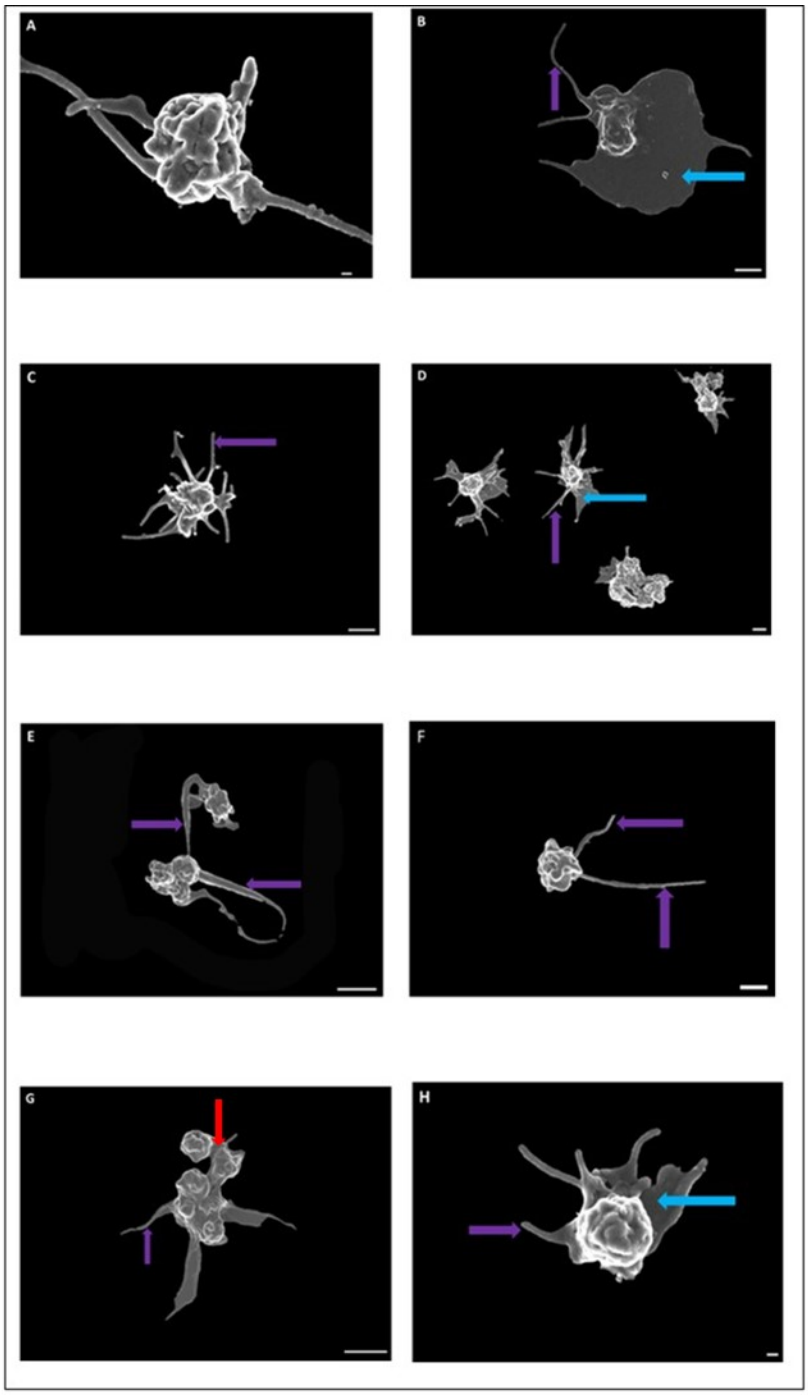

Figure 3: Scanning electron micrographs of control and x1 heavy metal exposed platelets. (A): Control, (B): Hg, (C): Ni, (D): Mn, (E) Hg+Ni, (F): Hg+Mn, (G): Ni+Mn and (H): Hg+Ni+Mn. A, C &D: Scale bars = 1µm; B: Scale bar=200 nm. Blue arrows= membrane spreading. Purple arrows= pseudopodia. Red arrow= platelet interaction.

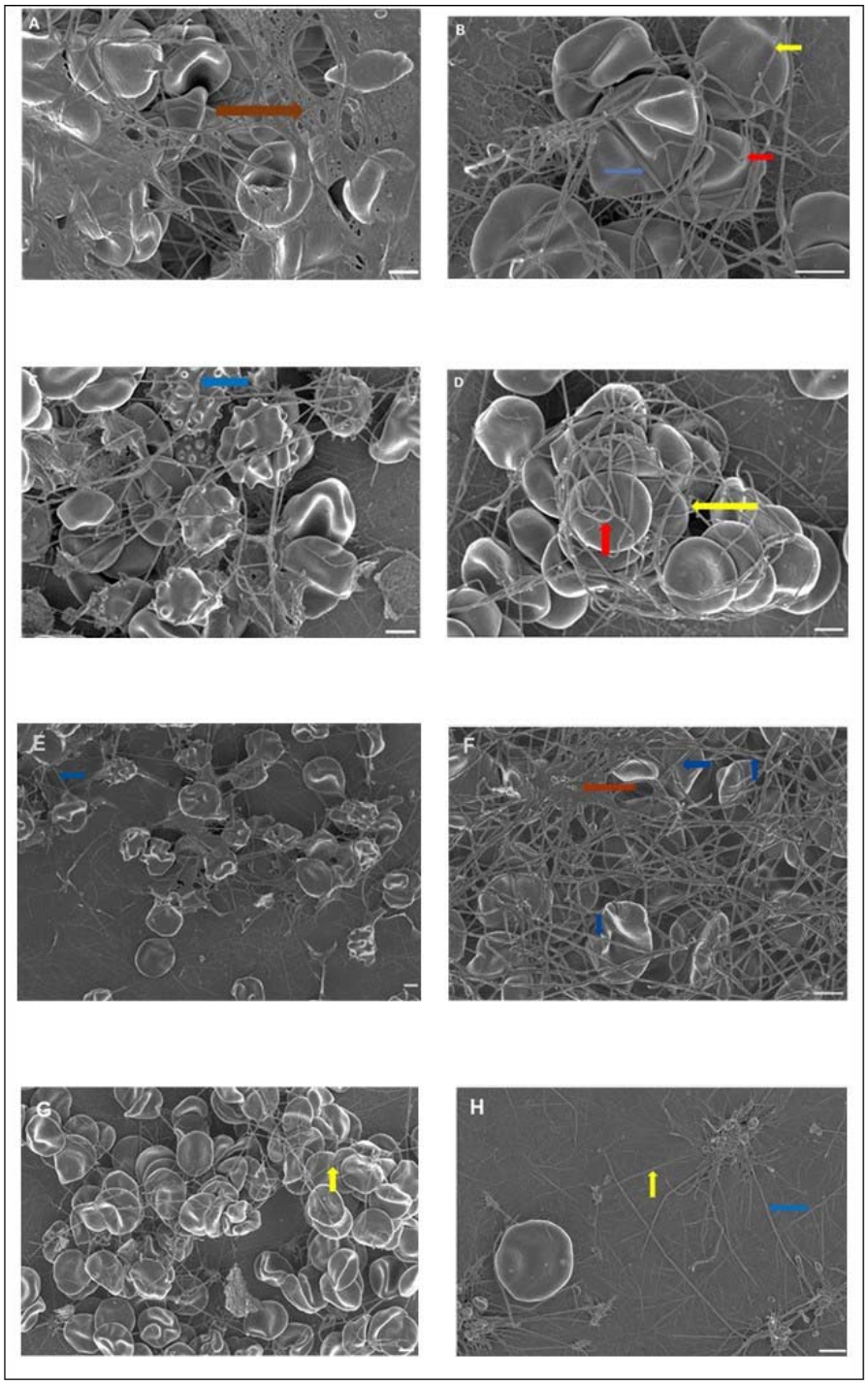

Figure 4: Scanning electron micrographs of whole blood with thrombin exposed to Hg, Ni and Mn, alone and in combination at concentrations at x1 showing fibrin network formation together with erythrocytes. (A): Control, (B): Hg. (C): Ni, (D): Mn, (E): Hg+Ni, (F): Hg+Mn, (G): Ni+Mn, (H): Hg+Ni+Mn. (A. C, D, F & H), Scale bars=2μm, (B, E & F) scale bars=1μm. Brown arrows=fibrin mesh. Yellow arrows = Thin fibres. Blue arrows= Thick fibres. Red arrows= Less taut fibrin fibres.

Confocal laser scanning microscopy

In the positive control, erythrocytes were exposed to Mellitin represented in Figure 5. Figure 5A represents the auto-fluorescence of the erythrocytes in the positive control. Figure 5B represents the positive Annexin V signal (indicated with green fluorescence) that was observed in the positive control. Figure 5C shows the transmission light image and Figure 5D shows the overlaid images. In the negative control group, no positive Annexin V signal was observed as shown in Figure 6A. Figures 6 B to H are images of Annexin V positive signal that were obtained in all the metal exposed groups, single (Hg, Ni and Mn), double (Hg+Ni, Hg+Mn and Ni+Mn) and triple combination (Hg+Ni+Mn). Phosphatidylserine flip positive cells were scattered throughout the samples.

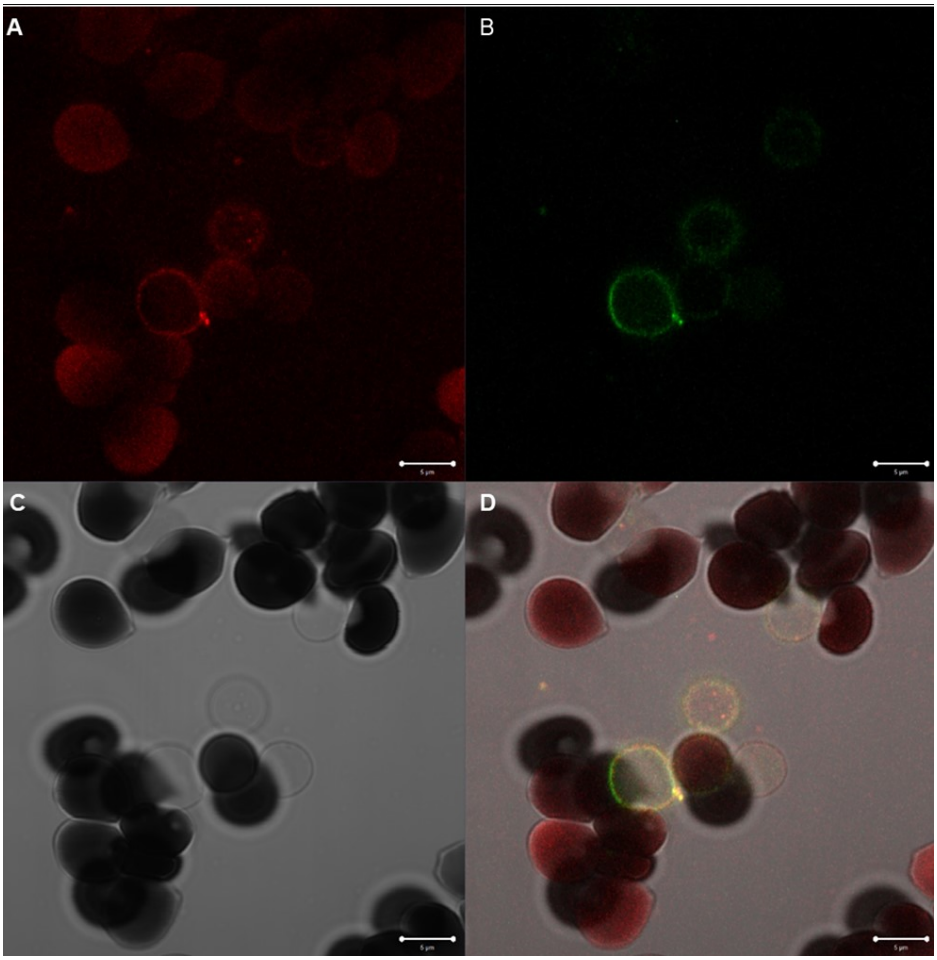

Figure 5: Confocal laser scanning micrographs of the positive control (A-D). Figures A: auto-fluorescence of the erythrocytes, B: Annexin V signal obtained, C: transmission light and D showing the overlay of the images (transmission, fluorescence and annexin V) (Scale bars: 5µm).

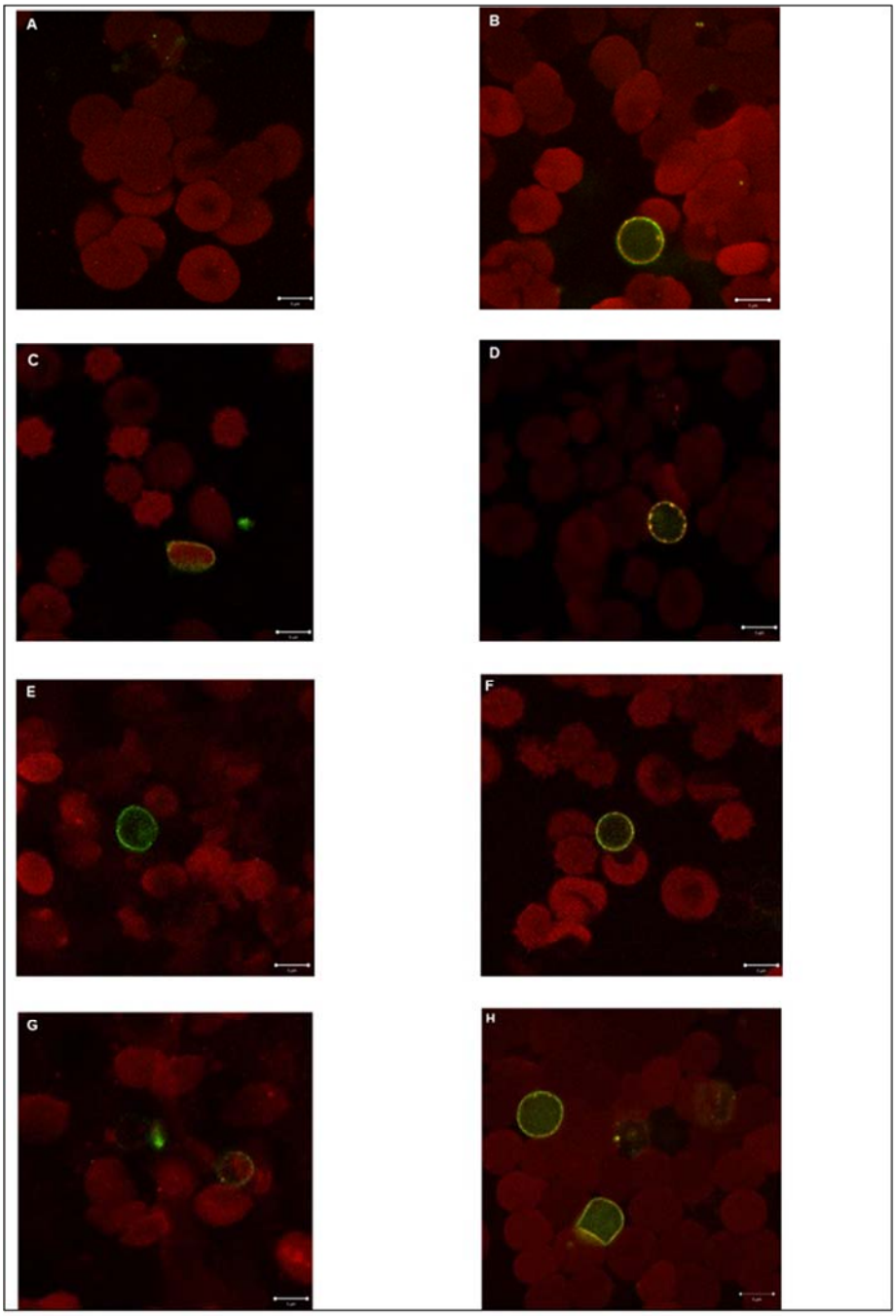

Figure 6: Phosphatidylserine exposure evaluation of erythrocytes subsequent to heavy metal exposure of Hg, Ni and Mn alone and in combination using the confocal laser-scanning microscope. (A): Negative control. (B- H) indicates the Annexin V positive erythrocytes after exposure to Hg (B), Ni (C), Mn (D), Hg+Ni (E), Hg+Mn (F), Mn+Ni (G) and Hg+Ni+Mn (H) (Scale bars: 5μm).

Discussion

Water, soil and air are natural resources that humans and animals survive on. These three resources need to be monitored to control and lower pollution levels and degradation of heavy metals. The production, processing as well as disposal of certain products that contain heavy metals is released into the immediate environment, which affects the soil, water and air quality. South Africa and the world at large have seen pressure coming from civil societies and governmental organizations to reduce air pollution [16] and, the banning or phasing out products and processes that rely specifically on Hg [17-18]. As a result, various companies are introducing innovative or upgraded air pollution control measures. Mercury is among several metals considered to be toxic at its lowest concentration [19]. It is thus very important for guidelines to be set with regards to the consumption of Hg. The WHO and the Joint FAO (Food and Agriculture Organization) Expert Committee on Food Additives (JECFA) established a safety intake of 1.6µg/kg bodyweight per week for CH_3Hg^+. The same value is recommended for pregnant mothers to protect the developing fetus from neurotoxic effects [20]. At concentrations of x10 000 the WHO safety limit Hg caused 68.132% haemolysis. In a similar study conducted by Janse van Rensburg *et al* in 2017 [21], the haemolysis percentage of Hg at x10 000 the WHO safety limit was 75.20% [21]. These findings correlate with the current study. There is a direct association between the concentration of Hg and percentage haemolysis of erythrocytes. Mercury plays a role in the propagation of oxidative stress thus resulting in the manifestation of cardiovascular diseases. The presence of Hg in erythrocytes inhibits superoxidase dismutase (SOD), catalase and glutathione peroxidase thus causing an increase in oxidative stress. This is achieved by Hg inhibiting antioxidant pathway by the development of ROS that bind to N-acetyl;cysteine (NAC) and glutathione (GSH) as a result of Hg having a high binding affinity to the thiol groups of NAC and GSH. A decrease in the content of GSH and other thiol containing redox molecules is due to the increase of Hg binding to GSH. Not only does this cause ROS accumulation but also causes enzyme inhibition activity and related biochemical pathways [22]. A relationship exists between increased Hg levels and the risk of developing cardiovascular diseases, pulmonary embolism, hypertension and the obstruction of vessels as seen in epidemiological studies [6].

Industries that make use of Ni have always faced environmental challenges such as air emissions, toxic effluents and waste. Airborne Ni compounds and $NiCl_2$ salts are carcinogenic to humans [23]. Exposure of Ni in occupational settings is still common [24]. The Ni plating industry and the coin industry makes use of high concentrations of Ni sulphate and $NiCl_2$ and if in contact with the skin it results in hand eczema [25]. At x10 0000 the WHO safety limit the haemolysis observed was

minimal at (1.66%). In a study conducted by Nielsen, 1999 [26], patients with eczema on their hands and Ni allergies submerged a finger into low Ni concentrations. The results showed extensive increase in the formation of vesicles and increase in blood flow with a comparison to a group that submerged their fingers in water. The Ni concentrations further caused inflammation and skin changes on patients who had sodium lauryl sulphate (SLS) treatment on the forearm skin [26] Noticeable effects of Ni- induced toxicity are seen with concentrations above 1000mg [27]

The WHO safety limit of Mn is 400 µg/L. The tolerable daily intake (TDI) is 60 µg/kg of body weight, based on the upper range value of Mn intake of 11000 µg/day [28-29]. As much as excessive consumption of Mn can lead to toxicity, deficiency of Mn can result in adverse effects. Manganese metalloenzyme SOD prevents lipid peroxidation. Lipid peroxidation is the process by which lipid oxidative degradation occurs. Radicals scavenge electrons from lipids within the cell, resulting in cellular damage. Manganese dependant enzymes prevents the process of lipid peroxidation by superoxide radicals [30-31]. Manganese can cause vasodilation of the vessels, thus causing decreased blood pressure following high administration of Mn [32-33].

It is rare that metal toxicity is restricted to one metal. Most toxicity studies of heavy metals are focused on one metal at a time. Metals from industrial areas may be emitted into the air one at a time but if there are various companies in a surrounding area that emit different metals, those metals will be found in combination within the atmosphere. In this study Hg, Ni and Mn were combined to observe what effects, the double combination of these heavy metals has on erythrocytes. A synergistic effect is of great concern because this results in the combination of metals being toxic to a greater extent than the metal on its own.

The Hg+Ni double combination at x1-x1000 resulted in no haemolysis. As shown in Table 4, at x1 the WHO safety limit the single metal Hg caused haemolysis, but Ni caused no haemolysis. These findings together with that shown in Table 4 indicate that the two metals have an antagonistic effect on each other. The same observations are seen at x10, x100 and x1000 the WHO safety limit. At x10000 WHO safety limit, the combination of the two metals result in haemolysis and caused a synergistic effect. A study on synergistic effect of Ni and Hg on fatty acid composition in the muscle of fish conducted by Senthamilselvan, 2016 [34] found that at high concentrations of 0.8, 2.0 and 4.0mg/L of Hg alone, Ni alone and Hg+Ni combination caused a decrease in fatty acid composition [34]. Mercury is a toxic and inhibitory metal in nature whilst Mn is an enhancer of growth at recommended WHO safety limits. The double combination of Hg+Mn yields no haemolysis at x1-x1000 concentrations respectively. At concentrations of Mn above the

WHO safety limit alone or in combination can be toxic. The results obtained from the combination of Mn+Ni showed that at x1 the WHO safety limit, there was no haemolysis observed and the interaction between the two metals was antagonistic. At x10-x10 000 the WHO safety limit, haemolysis was observed and the interaction between the metals at the various concentrations respectively yielded a synergistic effect. The interaction of the triple combination (Hg+Ni+Mn) of the metals only caused haemolysis at x1 and x10 000 the WHO safety limit. At x10, x100 and x1000 there was no significant haemolysis observed. A dosage dependent increase in haemolysis was not observed. The interaction at x1 and x10 000 was both synergistic. The percentage haemolysis at x10 000 the WHO safety limit was at 58%. To our knowledge, there is no literature available on the effect of Hg, Ni and Mn in combination on humans or animals.

Erythrocytes are extremely sensitive cells and aid in being a component of the body's health indicator [2]. The distribution of heavy metals causes adverse effects on blood homeostasis thus resulting in changes and damages to erythrocytes [21]. The normal functioning of erythrocytes is primarily dependent on the cell's intact membrane. Erythrocytes exposed to Hg x1 alone and in combination caused the erythrocytes to change from the normal biconcave shape to echinocytes (spike like nodules). In a study conducted by Lim, 2010 [35] exposure of Hg at 0.25μM to erythrocytes altered the biconcave shape into echinocytes and further into spherocytes depending on the length of exposure. Erythrocytes change into spherocytes by extensive loss of membrane surface due to the shape buffering capacity being lost thus resulting in a new default shape and formation of vesicles [35] Exposure of erythrocytes to Ni x1 caused cells to change from the typical biconcave morphology to cell blebbing (bulging appearance). With increase in concentration more cell blebbing was observed. A study done by De Luca, 2007 [36] indicated that exposure of $NiCl_2$ to erythrocytes caused morphological changes. The erythrocytes appeared spherical and echinocytes were also observed [36]. Manganese exposure caused cell blebbing (bulging appearance) to erythrocytes. In a study conducted by Chandel, 2016 [37] rats treated with Mn had distorted erythrocytes and it was dose-dependent, therefore suggesting that the metal lead to both quality and quantity decline in erythrocytes [37]. From the results obtained in the current study, Mn alone and in combination x1 caused changes to the erythrocytes. The cells changed from the normal biconcave shape to cell blebbing (bulging appearance). There was an increase in deformation of erythrocytes with an increase in concentration. The interaction of the three metals (Hg+Ni+Mn) caused cell blebbing (bulging appearance) of erythrocytes as seen in Figures 2H of the combination of metals. All erythrocytes exposed to the metals were distinctively different from

the control thus suggesting that heavy metal exposure does cause morphological changes. Increase in heavy metal concentration causes more damage to the cells.

Platelets from whole blood exposed to Hg x1 were seen to differ in morphology to the control platelets. An increase in pseudopods and membrane spreading was observed as well as the presence of a rough membrane surface. These findings are similar to those observed by Janse van Rensburg, 2017 [21] where erythrocytes exposed to Hg at varying concentrations appeared to have the same morphology as seen in this study [21]. Platelets from whole blood exposed to Ni became over activated with an increase in pseudopodia. With increased exposure of metals, multiple pseudopodia were observed, and platelets aggregated to form a clot. Manganese exposure caused the platelets to have increased pseudopodia, membrane spreading and activation. The combination of metals (Hg+Ni, Hg+Mn, Mn+Ni and Hg+Ni+Mn) also showed platelets changes, formation of increased pseudopodia, membrane spreading, highly activated platelets and platelet interaction. Interaction of the activated platelets was observed and shown in Figure 3G. The changes observed in platelets are due to the generation of reactive oxygen and nitrogen species, they are correlated with an increase in lipid peroxidation and carbonyl groups [38]. Critical signal regulating platelet activity has been suggested to be caused by ROS. Activation of platelets was observed in all experimental groups with Hg, Ni and Mn causing more changes to platelet morphology. All metals caused platelet activation.

One of the most important structural components of coagulation is fibrin fibres. The collaboration of fibrin and thrombin plays a major role in blood clotting [39]. Thrombin enables the conversion of factor XIII to factor XIIIa and the conversion of fibrinogen to fibrin [1,40]. The fibrin fibres form horizontal and vertical cross-linkages as seen from the images obtained in Figure 4. The addition of thrombin to the whole blood entraps the erythrocytes within a mesh of fibres (Figure 4A). It is important to note that the erythrocytes of healthy individuals keep their biconcave shape and do not fold around the fibrin fibres. Nonetheless, for the duration of systemic inflammation and conditions linked with the enhancement of eryptosis, the erythrocytes fold around the fibres losing their shape. This demonstrates vulnerability of the membrane. Typical characteristics of fibrin network usually encompasses particularly major thick fibres with a few thin fibres dispersed amongst them [41-42]. Figure 4F exhibits fibres and fibre aggregates thus increasing clot formation [43]. The presence of heavy metals causes the production of ROS which result in the indirect activation of platelets because the ROS scavenge nitric oxide [44]. The construction of the fibrin network has been shown to influence the process of fibrinolysis, and the breakdown process of fibrin fibres. In a study conducted by Collet, 2000 [45] fibrin fibres that are highly compacted and

composed of thin fibrin fibres breaks down slowly than the fibres with a loose fibrin fibre formation that is composed of thicker fibrin fibres [45]. From the results obtained erythrocytes become entrapped within the fibrin mesh together with the platelets as seen in Figure 4A. Thick and thin fibrin fibres are seen around the cells. Fibrin network forms a mesh structure in and around the erythrocytes. An increase in fibre thickness and fibre aggregation increases the formation of a clot [43]. In a study conducted to determine the effect of fibrin structure on fibrinolysis, it was determined that fibrin structure contributes to the regulation of the fibrinolytic rate. As the fibrin fibre decreases in size, the fibrinolytic rate also decreases. The structure of fibrin was altered by changing the ratio of thrombin to fibrinogen. Thin fibrin causes decreased fibrinolytic rate [46].

The exposure of PS on the outer leaflet of the erythrocyte's membrane is seen in Figure 5 B, E and F. In all three Figures Hg alone and in combination to Ni or Mn causes PS flip and exposure onto the cell's membrane surface. Eryptosis is characterized by cell shrinkage, cell blebbing and membrane scrambling resulting in the exposure of PS from the internal membrane of the cell surface. Eryptosis is triggered by a wide variety of causes such as oxidative stress, the presence of xenobiotic and endogenous substances, energy depletion, hyperosmolarity, increase in Ca^{2+}, antibiotics, ageing of the cells, inflammatory diseases such as Parkinson's disease, type two diabetes, increase in temperature and heavy metal exposure [47]. A study on employees occupationally exposed to mercuric vapour exhibited very high blood coagulation levels together with exacerbated thrombin generation levels [48]. Mercury induced procoagulation activation of erythrocytes might possibly influence the increase of cardiovascular diseases in human population. Zwaal, 1977 [49] conducted a study that concluded that modifications of erythrocyte membrane such as the exposure of PS and microvesicles formation is reported to cause erythrocytes to be procoagulant thus enabling erythrocytes to partake in thrombosis [49-50]. In a study conducted by Won-Hee, 2011 [48] Pb^{2+} caused an increased PS exposure and micro-vesicle generation in erythrocytes through the depletion of ATP. The results obtained from the confocal laser scanning microscope showed that exposure of Hg, Ni and Mn to erythrocytes caused a PS flip. Hg, Ni and Mn alone and in combination caused changes in erythrocyte morphology and this was associated with increased expression of Annexin V. Although all three metals have different mechanisms of causing toxicity, the result thereof is comparable to eryptosis.

Conclusion

Hg, Ni and Mn alone and in combination caused haemolysis, increased platelet activation, abnormal fibrin network formation and Annexin V positive signal. Heavy metals are environmental pollutants, toxins and oxidative stress promoters that are associated with a number of diseases that are disadvantageous to our health. Morphological alterations of erythrocytes, platelets and fibrin network was possibly due to ROS production, PS exposure and an increase in Ca2+. These factors all together can induce the formation of thrombus, which can result in CVD, including angina, myocardial infarction, stroke and venous thrombo-emboli. Cardiovascular disease affects many individuals and is becoming one of the greatest causes of deaths in the world.

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