Ultrastructural alterations of whole blood by copper, manganese and mercury metal mixtures using a chronic *in vivo* model of coagulation

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Highlights

•Contamination of drinking water by copper, manganese and mercury is of global concern.

•These heavy metals may contribute to thrombosis by causing haemostatic changes.

•This *in vivo* study compares the thrombotic potential of the metal groups *versus* a control.

•The heavy metals caused prothrombotic erythrocytes, platelets and fibrin networks.

•Chronic or high dose exposure to the heavy metals may contribute to thrombosis.

Abstract

Globally, contamination of drinking water by heavy metals is increasing and poses a potential hazard to human health. Data on heavy metal mixtures and their effects on thrombosis are limited. The objective of this study was to determine the *in vivo* effects that copper, manganese and mercury, alone and in mixtures, have on clotting potential. Forty-eight male Sprague-Dawley rats were divided into eight groups, dependent on the type of heavy metal/s administered. The dosages were calculated at X100 the World Health Organisation limits in drinking water and orally administered for 28 days, at the University of Pretoria in 2018. Heavy metal induced morphological alterations of erythrocytes, platelets and fibrin networks were evaluated, using scanning electron microscopy. The manganese and mercury mixture had the greatest thrombotic potential by inducing acanthocyte and echinocyte formation, generating highly activated platelets with spontaneous fibrin formation and forming a disorganised fibrin network. In conclusion, chronic or single high dosage exposure to these heavy metals can potentially induce or contribute to thrombosis.

Keywords: Drinking water contamination, heavy metals, morphology, scanning electron microscopy, thrombosis.

1. Introduction

Heavy metals are common global pollutants that can be found in drinking water across all continents and chronic heavy metal poisoning has become a global public health concern. There are 23 elements classified as heavy metals including copper (Cu), manganese (Mn) and mercury (Hg). In many countries across different continents, the concentrations of heavy metals in drinking waters are above international guidelines (Fernandez-Luqueno et al., 2013). The 2011 World Health Organisation (WHO) drinking water guideline values for Cu, Mn and Hg are 2000 µg/L, 400 µg/L and 6 µg/L, respectively. Coal combustion and gold mining in South Africa have contributed to an increase of metal contamination, including Cu, Mn and Hg, in water (Masekoameng et al., 2010; Sedibe et al., 2017; Yabe et al., 2010). Although they are considered pollutants, Cu and Mn are micro-nutritents needed for adequate biological functioning, while Hg has no known physiological role and is highly toxic (Sedibe et al., 2017). However, in excess all heavy metal ions are toxic and pose a human health risk due to toxic alterations to normal cellular- and tissue functioning (Jaishankar et al., 2014; Mudgal et al., 2010).

Although heavy metal toxicity has been acknowledged, exposure is still increasing globally. Heavy metals cause chronic adverse health effects such as cardiovascular disease (CVD) (Jaishankar et al., 2014). Cardiovascular disease (CVD) is an increasing global health problem (Alissa and Ferns, 2011). Central to disease development are changes in blood haemostasis associated with inflammation and oxidative stress, which results in thrombosis through a combination of hypercoagubility, a disturbance in blood flow and endothelial damage (Grandl and Wolfrum, 2018; Litvinov and Weisel, 2017; Roshan et al., 2011). Heavy metals increase reactive oxygen species (ROS) formation and decrease activity of anti-oxidant molecules such as glutathione (GSH) thus causing biochemical changes leading to free radical damage, lipid peroxidation, hyperglycaemia and hyperlipidemia, which could alter the coagulation profile of an individual and thus contribute to cardiovascular pathology (Roshan et al., 2011).

Erythrocytes, platelets and fibrin fibres are all important components of blood haemostasis with their own physiological and pathophysiological morphology and roles in the cardiovascular system (Table 1). Physiologically, erythrocytes and platelets have a low adhesiveness but ROS formation and oxidative stress can alter this (Cortese-Krott and Shiva, 2019). Reactive species mediate important physiological redox signalling pathways needed for normal cellular functioning (Cortese-Krott and Shiva, 2019; Nikolić-Kokić et al., 2010). Redox physiology is comprised of interactions between the erythrocytes, platelets and other circulatory factors. Erythrocytes and platelets both contain oxidant and anti-oxidant systems that keep them functional and capable of performing their primary functions (Table 1) (Cortese-

Krott and Shiva, 2019). However, substantial oxidative stress can cause anti-oxidant enzymes to lose activity and gain a pro-oxidative property, which consequentially produces more ROS, damaging other components of the circulation and oxidizing fibrinogen (Nikolić-Kokić et al., 2010). This oxidative stress burden can lead to a pro-thrombotic state with platelet hyperaggregability, increased fibre formation, increased microvesicle (MV) formation and erythrocyte membrane fragility and loss of deformability through phosphatidylserine (PS) externalisation (enhances aggregation) and the formation of spectrin/actin/ankyrin cross-links causing asymmetry of the lipid bi-layer (Cortese-Krott and Shiva, 2019; Nikolić-Kokić et al., 2010; Undas, 2014). Thus, the redox state of erythrocytes and platelets has profound implications and can possibly indicate the oxidative burden of an individual (Cortese-Krott and Shiva, 2019). Pathologically altered erythrocytes, platelets and fibrin networks (Table 1) are thus procoagulant (Litvinov and Weisel, 2017).

Table 1: The primary function and physiological/pathophysiological morphology of erythrocytes, platelets and fibrin networks.

Circulatory components	**Primary function**	**Physiological morphology**	**Pathophysiological morphology**
Erythrocytes	Transportation of oxygen and mediation of carbon dioxide (Nikolić-Kokić et al., 2010)	Discoid, bi-concave, smooth membrane, ± 7 µm in diameter (Gyawali et al., 2015)	Spike formation, blebbing, size alterations, shape alterations, haemolysis (Lang et al., 2012)
Platelets	Prevention of blood loss by forming haemostatic thrombi (Li et al., 2010)	Resting, non-adherent phase: Discoid, no pseudopodia (Li et al., 2010; Posch et al., 2013)	Pseudopodia formation, spreading, aggregate formation (Posch et al., 2013)
Fibrin networks	Clot formation (Kattula et al., 2017)	Branched network of straight thick and thin fibres under tension (Weisel and Litvinov, 2017)	Disorganised, dense network, fibrin clumping, sticky thick fibres, many thin fibres (van Rooy et al., 2015)

In an *ex vivo* human model, Cu, Mn and Hg have been shown to bind anti-oxidant molecules such as GSH and act as catalysts of the Fenton reaction inducing radical formation and altering blood haemostasis (van Rensburg et al., 2019). However, a limitation of such studies is that the consequence of absorption, distribution, metabolism and excretion is not taken into account. Therefore, the widely used Sprague-Dawley (SD) rat model was used to address this limitation (Arbi et al., 2017; Kenston et al., 2018; Venter et al., 2017). Dosage based studies do not necessarily correlate with the effects of environmental exposure and for this reason, the WHO limit was selected and converted to a rat equivalent dosage (Nair and Jacob, 2016). This strategy has previously been successfully implemented in *ex vivo* and *in vivo* studies and is advantageous as the effect of several metals at several fold of the established limits can be

evaluated (Arbi et al., 2017; van Rensburg et al., 2019; Venter et al., 2017). The aim of this study was to investigate the potential thrombotic effects of exposure to Cu, Mn and Hg, alone and as part of mixtures, in a chronic exposure SD rat model on the ultrastructural morphology of erythrocytes, platelets and fibrin networks with scanning electron microscopy (SEM).

2. Materials and Methods

2.1 Metal preparation

2.1.1 Chemical compounds

Copper (II) sulphate ($CuSO_4.5H_2O$, purity: 98%), manganese (II) chloride ($MnCl_2.4H_2O$, purity: 99%) and mercuric chloride powder ($HgCl_2$, purity: 99%) were dissolved in distilled, deionised water (ddH_20). All metal compounds were purchased from Sigma-Aldrich, South Africa.

2.1.2 Dosage calculations

The final daily dosage solutions for the metals are presented in Table 2. The concentrations chosen were based on and were X100 greater than the 2011 WHO drinking water guideline values, for each respective metal. The calculated concentrations were based on a 60kg adult drinking 2L of water a day. A 150g rat equivalent dosage was then calculated (Nair and Jacob, 2016).

Table 2: Dosage calculations for the oral gavage solutions of the experimental groups.

	Cu	Mn	Hg
WHO limit (mg/L)	**2.0**	**0.4**	**0.006**
WHO limit X 100 (mg/L)	200.0	40.0	0.6
Molecular weight: metal (g/mol)	63.5	54.9	200.6
Metal ion concentration (mM)	3.147	0.728	0.003
Molecular weight: metal-salt (g/mol)	249.7	197.9	271.5
Metal salt concentration (g/L)	0.786	0.144	0,0008
Daily intake (g/2L)	1.572	0.288	0.0016
Human (60 kg) daily intake (mg/kg)	26.2	4.8	0.027
Rat (150 g) intake (mg/kg)	161.5	29.6	0.17
Dosage solutions (mg/mL)	**48.5**	**8.9**	**0.05**

Note: Combinational groups require the same (mg) of each respective metal

2.2 Sprague-Dawley rat model

2.2.1 Experimental animals

Six week old male SD rats (150 g) were obtained from the University of Pretoria's Biomedical Research Centre (UPBRC). Female rats were excluded to avoid the thrombotic effect that the female hormone, oestrogen, has on the clot structure (Swanepoel et al., 2017).

2.2.2 Housing conditions

The animals were housed in conventional cages complying with the sizes described in the South African National Standards (SANS) 10386:2008 recommendations. A room temperature of 22°C (±2°C); relative humidity of 50% (±20%) and a 12-hour light/dark cycle were maintained during the entire study. The rats were housed in pairs in cages with autoclaved pinewood shavings as bedding material. White facial tissue paper was added for enrichment according to standard procedures at the UPBRC. The rats were acclimatized for 7 days prior to the start of the experimentation period. The rats were weighed bi-weekly to observe any sudden change in weight and behaviour of the rats was also monitored daily.

2.2.3 Experimental design

Forty-eight male rats were included and were randomly divided into eight groups (six rats per group), as follows: control, Cu, Mn, Hg, Cu + Mn, Cu + Hg, Mn + Hg and Cu, Mn + Hg. The control group received a saline solution, 0.9% sodium chloride (NaCl), only. The seven experimental groups received the metal mixtures assigned, at the final concentrations presented in Table 2. All rats were administered 0.5 mL of the respective solutions through daily oral gavage, for 28 days.

2.2.4 Termination

The rats were terminated on day 28 via isoflurane (general anaesthesia) overdose and cardiac puncture, according to the standard methods employed by the UPBRC.

2.2.5 Blood collection

Blood was collected in EVAC citrate tubes containing 3.2% sodium citrate. All experimental protocols complied with the requirements of the University of Pretoria's Animal Ethics Committee (AEC), ethical clearance number: 6/2019.

2.3 Scanning electron microscopy

For morphological studies of erythrocytes, platelets and fibrin networks, citrated whole blood was used. A volume of 10 µL of whole blood was used to make smears on 10 mm round glass cover slips, with and without the addition of 5 µL of human thrombin (20 U/mL). After making the smears, the cover slips were incubated for 10 minutes at room temperature and then washed in a 0.075 M phosphate buffer solution (PBS) for 20 minutes. The samples were then fixed in a 2.5% glutaraldehyde/formaldehyde (GA/FA) solution (5 mL 0.075 M PBS – pH 7.4, 1 mL 25% GA, 1 mL 25% FA and 3 mL ddH_20) for 30 minutes and were then washed thrice in the 0.075 M PBS. The samples then underwent secondary fixation in 1% osmium tetroxide for 15 minutes and were then washed thrice again in the 0.075 M PBS. The samples were then dehydrated by using an increasing serial dehydration step with 30%, 50%, 70% and 90% ethanol (EtOH), followed by three changes of 100% EtOH. The 100% EtOH was removed and a 100% hexamethyldisilazane (HMDS) was added, for 30 minutes. After discarding, an additional 100 µL of HMDS was placed on the cover slips and the samples were incubated at room temperature to dry. These samples were then mounted on aluminium stubs, coated by carbon evaporation and viewed with the Zeiss Ultra PLUS FEG SEM and Zeiss Crossbeam 540 FEG scanning electron microscope at 1 kV.

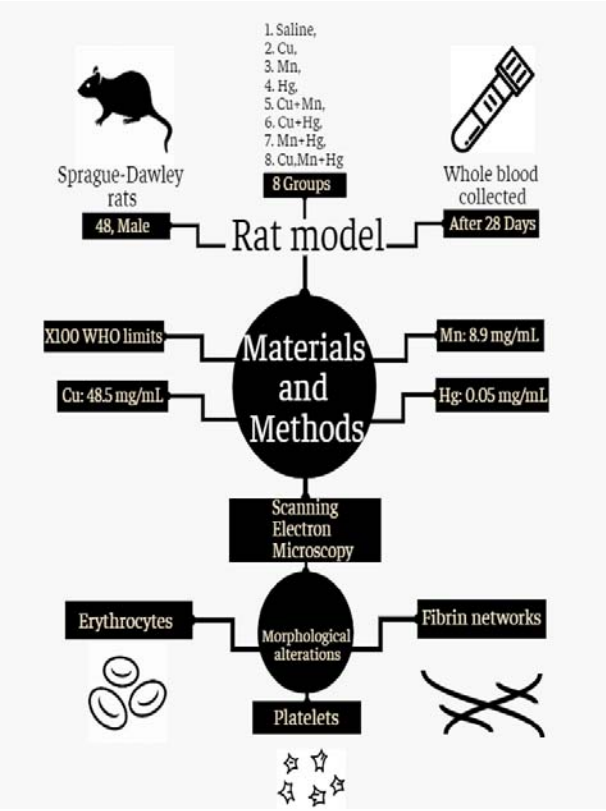

Figure 1: Diagram illustrating the methodology of the study.

3. Results and Discussion

Environmental heavy metals are commonly found in mixtures and these mixtures pose a risk to human health (Kenston et al., 2018). In areas surrounding mining and coal combustion facilities, metal levels often exceed the permissible limits (Kamunda et al., 2016; Okedeyi et al., 2014). Metal toxicity has been widely investigated, but few studies have investigated the *in vivo* effects of metal mixtures on the vascular system and specifically the effect of exposure on the ultrastructure of erythrocytes, platelets and fibrin networks. In this study, the effects of the metals Cu, Mn and Hg, alone and as part of metal mixtures, at X100 the WHO limit for drinking water on the erythrocyte, platelet and fibrin network morphology was investigated. This study represents a model of chronic exposure where 28-day exposure in the SD rats represents three years chronic human exposure (Nair and Jacob, 2016). The results yielded varying morphological alterations dependant on cellular targets and metal type, which can be attributed to mode of action and concentration of the metals used.

3.1 Erythrocytes

In healthy individuals, most erythrocytes exhibit a typical discocyte shape where about 1% of erythrocytes are abnormally shaped (Swanepoel and Pretorius, 2012). Representative erythrocyte micrographs acquired from whole blood smears without the addition of thrombin are shown in Figure 2. In Figure 2A, an erythrocyte of the control group is depicted and demonstrates the biconcavity and smooth membrane of a typical discocyte. The erythrocytes of the experimental groups are demonstrated in Figures 2B - H. Overall, different forms of erythrocyte membrane deformity with a loss of biconcavity were demonstrated in all the experimental groups, exhibiting scalloped borders (dashed white arrow) – B and H, membrane projections (thick white arrow) – B, C, D and H and nodule-like spicules (thin white arrow) – E to H. However, the Mn + Hg group demonstrated the most structural changes to erythrocytes, as compared to all other groups. A summary on the morphological findings of the erythrocytes is presented in Table 3.

Table 3: Summary of the morphological changes to erythrocytes following metal exposure.

	Membrane disruption	Shape alteration	Scalloped borders	Membrane projections	Nodule-like spicules
Cu	+	-	+	+	-
Mn	+	+	-	+	+
Hg	++	++	-	++	++
Cu + Mn	++	++	+	++	+
Cu + Hg	++	++	-	++	++
Mn + Hg	+++	++	+	++	++
Cu, Mn + Hg	++	++	+	++	++

–: no or minimal; +: slight; ++: moderate; +++ and severe alterations

In a dosage dependent study the effects of a 60 day Mn exposure on the morphology of Wistar rats' erythrocytes were investigated. Dosages of 50, 100 and 150 mg/kg induced the formation of distorted erythrocytes (similar to Figure 2B and C), increased acanthocyte prevalence (similar to Figure 2D and E) and induced the formation of both acanthocytes and echinocytes (similar to Figure 2H), respectively (Chandel and Jain, 2016). The highest dosage used was lower than the current studies dosage (22.5 mg vs. 29.6 mg / 150g rat), but the experimental period was longer (60 vs. 28 days). A reduction in erythrocyte and platelet counts was also noted, indicating that Mn inhibits the haemopoietic system in addition to having direct effects on erythrocyte membrane morphology (Chandel and Jain, 2016).

Two types of abnormal erythrocytes can be identified in the present study – acanthocytes: deformed erythrocytes with few, irregularly spaced membrane projections or spicules and echinocytes: symmetrical, deformed erythrocyte with many regularly spaced membrane projections or spicules (Pretorius et al., 2016; Swanepoel and Pretorius, 2012). All the experimental groups demonstrated erythrocyte deformation with acanthocytes being demonstrated in Figures 2D, E and H and echinocytes being demonstrated in Figures 2F, G and H. The increase in echinocyte formation is associated with risk for thrombosis, as a high prevalence of these types of erythrocytes has been identified in stroke patients. Spicule formation is due to the outer membrane leaflet of the bi-lipid layer of the erythrocyte membrane expanding relative to the inner membrane, whilst cavities occur when there is contraction. These morphological alterations can occur from the combination of force and shear stress changes due to cell membrane damage from proteolysis or oxidation (Swanepoel and Pretorius, 2012).

The effect of 1 hr Hg exposure at 0.25, 0.5 and 1 mg/kg on SD rats was investigated. An increase in PS exposure and thrombosis, as well as inhibition of flippase and activation of scramblase enzymes was demonstrated. The proposed Hg mediated thrombotic effect is due to PS externalisation, MV generation due to thiol and ATP depletion and increased Ca^{2+} levels (Lim et al., 2010). Although not conclusive, indications are that Hg mediated depletion of ATP, may occur to a greater degree than for Cu and Mn, where the latter metals cause the catalyses of the Fenton reaction, induce ROS formation and/or GSH depletion (van Rensburg et al., 2019). Manganese also increases Fe (II) levels from Fe (III), which further exacerbates ROS production and lipid peroxidation (Chandel and Jain, 2016). The Hg combinations increased erythrocyte toxicity, with the greatest changes being observed for Mn + Hg and the triple combination group, indicating that the ROS inducing effects of Mn enhance the toxicity of Hg.

3.1.1 Scanning electron micrographs: Erythrocytes

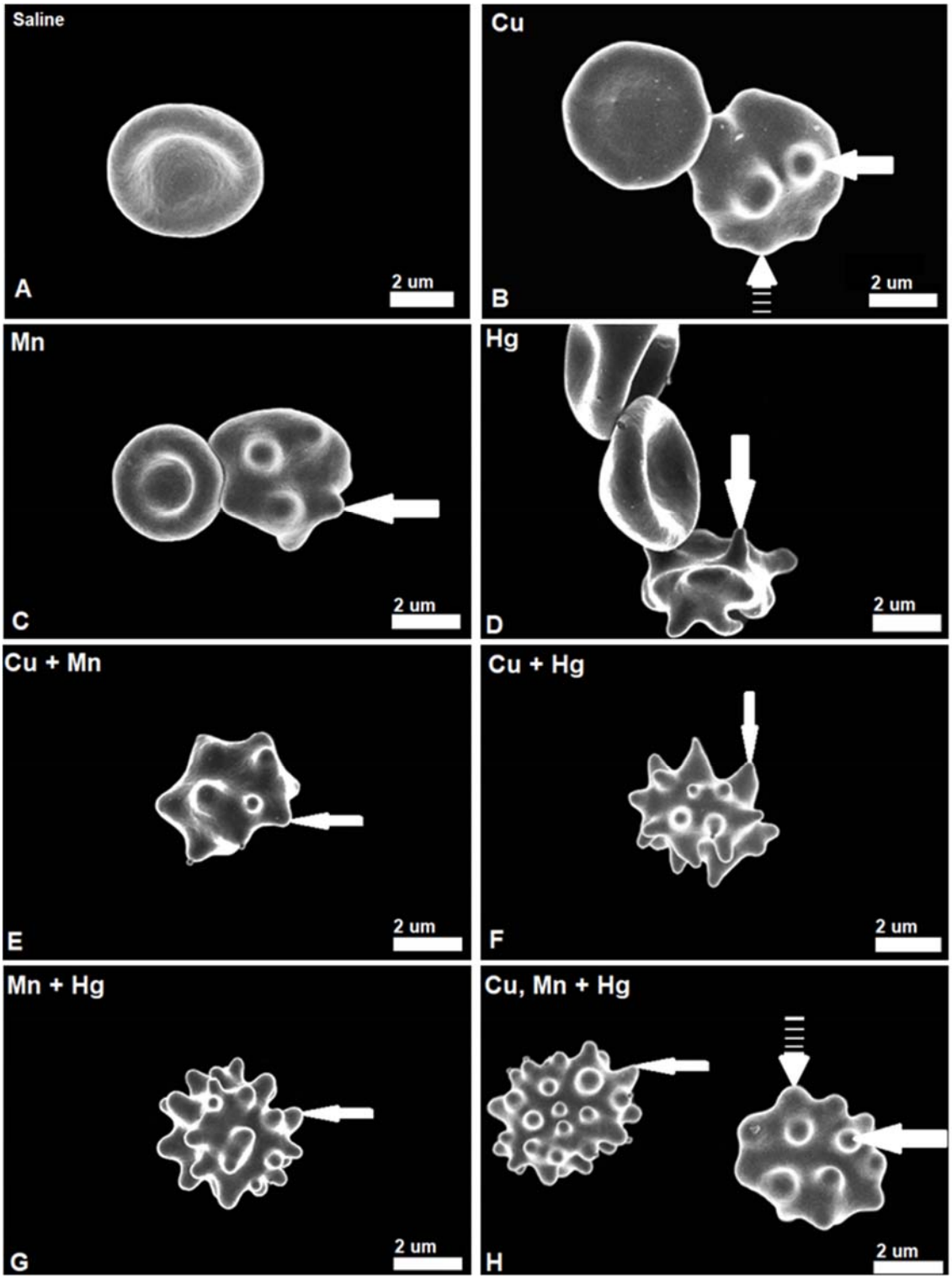

Figure 2: SEM micrographs of whole blood without thrombin of SD rats exposed to Cu, Mn and Hg metal mixtures showing erythrocyte morphology; Scale bar = 2 µm. **A**: Control, **B**: Cu, **C**: Mn, **D**: Hg, **E**: Cu + Mn, **F**: Cu + Hg, **G**: Mn + Hg, and **H**: Cu, Mn + Hg. **Thin white arrows**: nodule-like spicules; **thick white arrows**: membrane projections and **dashed white arrows**: scalloped border.

3.2 Platelets

3.2.1 Scanning electron micrographs: Platelets

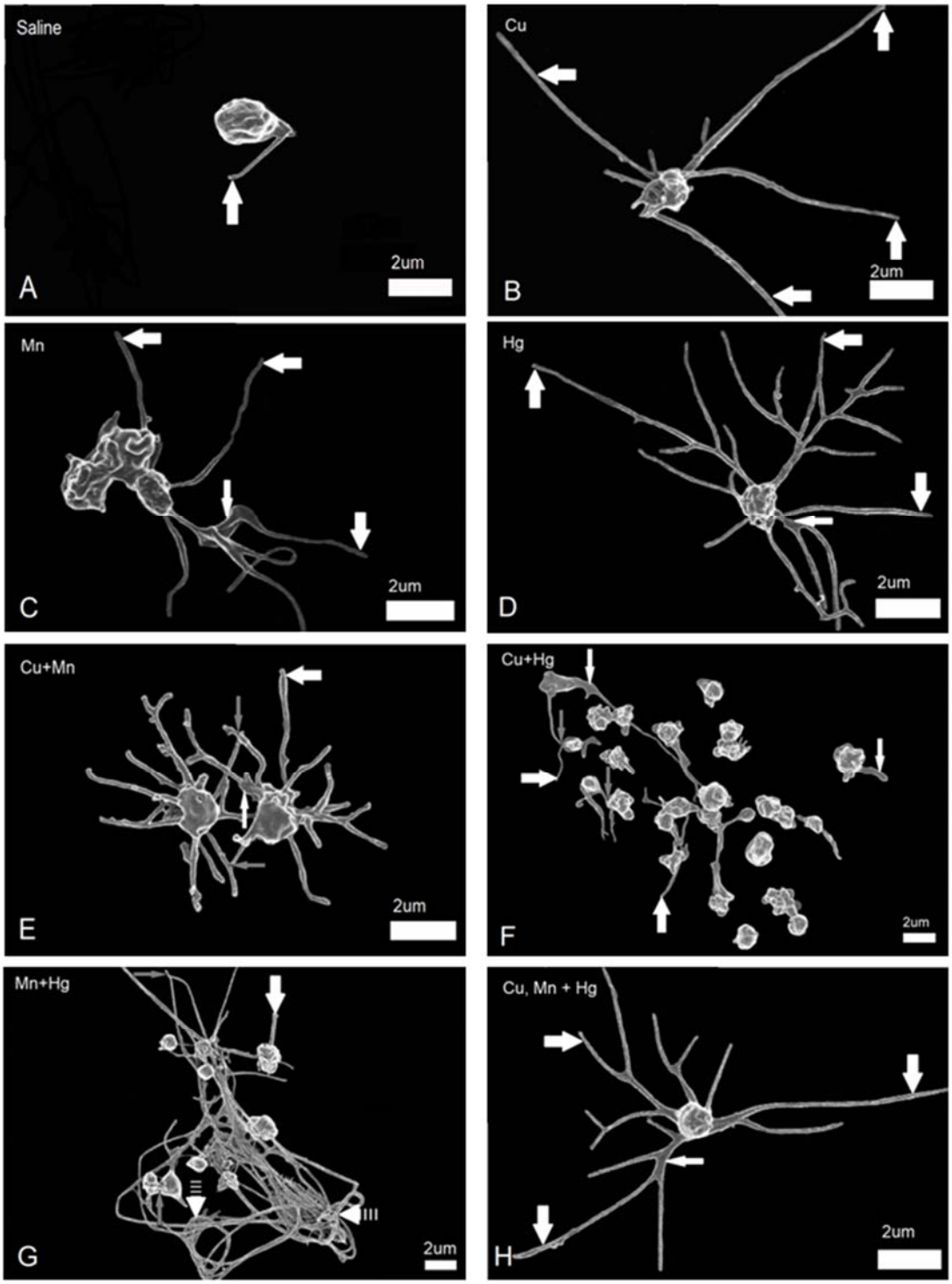

Figure 3: SEM micrographs of whole blood without thrombin of SD rats exposed to Cu, Mn and Hg metal mixtures showing platelet morphology; Scale bar = 2 µm. **A**: Control, **B**: Cu, **C**: Mn, **D**: Hg, **E**: Cu + Mn, **F**: Cu + Hg, **G**: Mn + Hg, and **H**: Cu, Mn + Hg. **Dashed white arrows**: platelet aggregates; t**hin grey arrows**: platelet interaction; t**hick white arrows**: pseudopodia and **thin white arrows**: platelet spreading.

Representative platelet micrographs acquired from whole blood smears without the addition of thrombin are shown in figure 3. In figure 3A, the control group platelet demonstrates a discoid morphology with a single pseudopod (thick white arrow). The platelets of the experimental groups are demonstrated in figures 3B – H. Overall, the metals induced platelet activation through an increase in pseudopodia (thick white arrow) – B to H, platelet spreading (thin white arrow) – B to F and H, platelet-to-platelet interactions (thin grey arrow) – E to H and platelet aggregate formation (dashed white arrow) – F and G. The Mn + Hg group demonstrated the highest degree of platelet aggregation was and spontaneous fibrin formation, which can lead to the development of thrombosis. A summary of the morphological features of platelets following exposure to the metals is presented in Table 4.

Table 4: Summary of the morphological changes to platelets following metal exposure.

	Increased pseudopodia	Membrane spreading	Platelet interactions	Platelet aggregation	Platelet activation
Cu	+	-	-	-	+
Mn	+	+	-	-	+
Hg	++	-	-	-	++
Cu + Mn	++	+	++	-	++
Cu + Hg	+	+	++	+	+
Mn + Hg	+	-	+	++	++
Cu, Mn + Hg	++	-	+	-	++

–: no or minimal; +: slight; ++: moderate; +++ and severe alterations

Small increases in intracellular calcium (iCa^{2+}) can lead to platelet activation, but mediation of this occurs through Ca^{2+} ATPases present in the endoplasmic reticulum (ER) and plasma membrane that pumps Ca^{2+} back into the ER and out of the platelets, respectively (Varga-Szabo et al., 2009). Oxidative stress can damage platelet membranes and disrupt the mediated Ca^{2+} haemostasis resulting in an increased iCa^{2+} that activates the mitochondrial permeability transition pore (MPTP), resulting in platelet activation (Jobe et al., 2008).

Copper (II) ions, homocysteine and hydrogen peroxide form physiologically relevant concentrations of hydroxyl radicals, but together with thrombin result in dramatic changes to morphology and extensive shedding of membrane particles as compared to human platelets activated by thrombin alone. However, the inclusion of cyclosporine A, a cyclophilin inhibitor, results in a reversal of platelet activation with platelet morphology appearing normal. In this study, wild type and cyclophilin D knockout (CypD -/-) mice were used and the morphology of platelets exposed to thrombin and ROS were compared. For CypD -/- activation was reduced, indicating that CypD, a MPTP enhancer, is essential for platelet activation. Activation of MPTP, increases high-level fibrinogen retention, PS externalisation and the procoagulant activity of platelets (Varju et al., 2018). In the present study, intracellular H_2O_2 levels are likely to be determining the levels of hydroxyl radical formation via the Fenton reaction. Further depletion

of anti-oxidant elements such as GSH and ATP and the inhibition of anti-oxidant enzyme activity will advance this effect, with Cu + Mn and Mn + Hg possibly having the greatest thrombotic potential.

3.3 Fibrin networks

The addition of thrombin to whole blood allows for the determination of the heavy metal effects on the formation and structural properties of the formed fibrin network. Representative micrographs of the fibrin networks acquired from whole blood smears prepared with the addition of thrombin are shown in Figure 4. In Figure 4A, the fibrin network of the control group is depicted with very few thin (thin white arrows) and numerous thick (thick white arrows) fibres that are straight and taut and form an organised network. Normal erythrocytes are also present within the network. In Figure 4B – H, the fibrin fibre networks of the experimental groups all demonstrate deformed erythrocytes (thin light grey arrow). Some experimental groups demonstrated features of a normal, healthy network with numerous, straight thick (thick white arrow) and some thin fibres (thin white arrow) forming an overall organised, taut network with lateral aggregates (thick black arrow) also occurring – B, C. However, overall all the experimental groups showed some degree of morphological alterations to the fibrin networks, including less taut fibres (thin light grey arrow) – B to H, net-like coverings (thin black arrow) – B, E and F, fibrin clumping (black ring) – B, D, F and G, fused areas (thick dark grey arrow) – C, D and G, broken fibres (white ring) – C, D and F, an increased number of thin fibres (thin white arrow) – E to G and a disorganised arrangement – E and G. The Mn + Hg and the Cu + Mn combinational groups demonstrated the highest degree of fibrin network structure alterations. A summary of the morphological changes to the fibrin networks is presented in Table 5.

Table 5: Summary of the morphological changes to fibrin networks following metal exposure.

	Increase in minor thin fibres	Less taut fibres	Net-like covering of minor thin fibres	Clumping of fibres	Fused areas of fibrin fibres	Broken fibres	Network type
Cu	-	+	+	+	-	-	Organised
Mn	-	+	-	-	+	+	Organised
Hg	-	++	-	++	+	+	Organised
Cu + Mn	+++	+++	++	-	-	-	Disorganised
Cu + Hg	++	+	++	++	-	++	Organised
Mn + Hg	+++	+	-	+	+	-	Disorganised
Cu, Mn + Hg	-	+++	-	-	-	-	Organised

–: no or minimal; +: slight; ++: moderate; +++ and severe alterations

3.3.1 Scanning electron micrographs: Fibrin networks

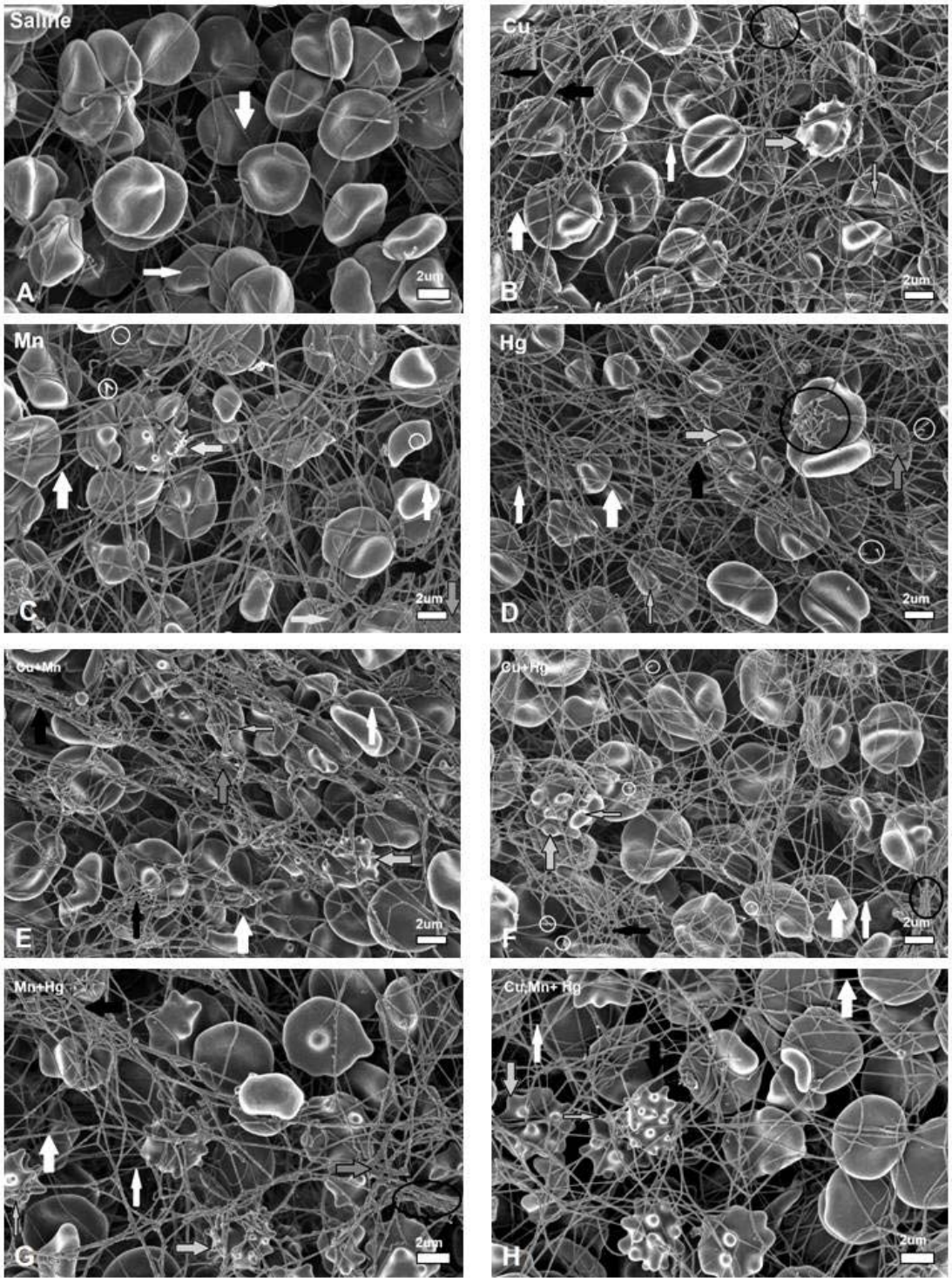

Figure 4: SEM micrographs of whole blood with thrombin of SD rats exposed to Cu, Mn and Hg, metal mixtures showing fibrin network morphology; Scale bar = 2 µm. **A**: Control, **B**: Cu, **C**: Mn, **D**: Hg, **E**: Cu + Mn, **F**: Cu + Hg, **G**: Mn + Hg, and **H**: Cu, Mn + Hg. **Black circle**: clumping of fibres; **white circle**: broken fibres; **thin black arrows**: net-like covering of thin fibres; **thick black arrows**: Lateral aggregates of fibrin fibres; **thick dark grey arrows**: fused fibrin fibre areas; **thin light grey arrows**: bending, less taut fibres; **thick light grey arrows**: deformed erythrocytes; **thin white arrows**: thin minor fibres and **thick white arrows**: thick major fibres.

The presence of metals in platelet rich fibrin of non-smokers and smokers has been identified. Relevant to the present study, Mn and Hg were present with Mn levels being increased in smokers. This study identified that the presence of increased metal levels may directly adversely affect fibrin network formation (Yaprak and Yolcubal, 2019). In an *ex vivo* study, using human blood, a reduction in the lysis potential of the clot was also observed for all combinations, especially Cu in combination with Hg as well as Mn alone (van Rensburg et al., 2019). In the present SD rat study, differences were observed, but generally, the fibrin networks that formed following exposure to Mn were disorganised while Hg formed organised fibrin networks. For the combination Cu + Mn the network was disorganised and had the greatest adverse potential of all the metal combinations. Differences between the *ex vivo* and SD model findings highlights the importance of factors such as selective uptake mechanisms, metabolism and excretion as processes that reduce toxicity.

The effects of reagents that mimic oxidative stress on fibrinogen function have been investigated. Malondialdehyde formation, that results in inter- and intra-cross-linking of proteins such as fibrinogen and carbonyl group formation, resulted in the formation of fibrin clots with thinner fibres as was observed for Cu + Mn and Mn + Hg (Stikarova et al., 2013). The presence of broken fibres indicates that, with coagulation, the formed fibres are fragile due to incomplete or poor fibre formation possibly due to oxidative modifications of fibrinogen. The presence of these broken fibres was most prevalent in the Mn + Hg group.

Clotting and the risk for thrombosis is a complex process and is a function of the cells, platelets and proteins involved, as well as the effects of endogenous factors, such as metal exposure on function and structure. Altered erythrocyte morphology, premature/rapid platelet activation and the formation of clots resistant to fibrinolysis increases the risk for thrombosis. For the single metals evaluated, Hg had the greatest adverse effect on erythrocyte and platelet morphology and fibrin network formation, while for the metal mixtures Mn + Hg had the greatest effect on all components with the highest degree of erythrocyte membrane disruption, platelet aggregation and fibrin network disorganisation and presence of minor thin fibres and Cu + Hg demonstrated the highest degree of disorganisation, presence of minor thin fibres as well as clumping of fibres and less taut, bent fibres in the fibrin networks. Interestingly, the triple combination had the least effect and this may be to antagonistic interactions between metals. The features mentioned here indicate an increase in toxicity of these groups and thus a potential increase in thrombosis.

In conclusion, all single and combination metal groups induced erythrocyte membrane deformation, platelet activation and fibrin networks with a more thrombotic morphology. The Mn + Hg group showed the highest degree of thrombotic effects with echinocyte formation,

platelet aggregation, spontaneous fibrin formation and a disorganised fibrin network with many thin fibres. This study has identified whole blood as a target to the metal ions Cu, Mn and Hg and demonstrates that chronic exposure to metal mixtures in drinking water can adversely affect blood haemostasis and thus overall health through an increased risk of CVD development. The formation of ROS, depletion of antioxidant elements and possibly other mechanisms of action such as induction of eryptosis, increased PS externalisation, increased iCa^{2+} levels and MPTP activation are possible factors for the morphological alterations noted and should be investigated in future studies.

4. Declaration of Interest

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the article.

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