Induction of hepatic portal fibrosis, mitochondria damage and extracellular vesicle formation in Sprague-Dawley rats exposed to copper, manganese and mercury, alone and in combination

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Abstract

Increased anthropogenic activity and subsequent environmental exposure to heavy metals induces the production of reactive oxygen species (ROS), which increases oxidative stress and the risk of associated diseases. The aim of this study, in a subacute model of toxicity, was to investigate the effects of copper (Cu), manganese (Mn) and mercury (Hg) alone and in combination on the liver tissue of male Sprague-Dawley rats, exposed orally to 100 times the World Health Organization's acceptable water limits of each metal. General histological alterations as well as ultrastructural changes were investigated using light microscopy and transmission electron microscopy (TEM) respectively. Exposure to Cu, Mn and Hg, alone and in combinations, caused hydrophic swelling of the hepatocytes, dilation of the sinusoids, formation of binucleated hepatocytes with an increased inflammatory cell accumulation at the portal triad. Increased collagen deposition with associated fibrosis was also observed. Evaluation of hepatocyte ultrastructure revealed mitochondrial membrane damage and inner membrane swelling especially for hepatocytes exposed to Mn. Extracellular vesicle (EV) formation was observed in the liver tissue of all exposed rats. Furthermore, increased damage observed for metal combinations was possibly due to synergism. In conclusion, Cu, Mn and Hg alone and as part of a mixtures causes cellular damage, inflammation and fibrosis increasing the risk of associated diseases.

Key words: Copper, manganese, mercury, liver, fibrosis, mitochondria damage, extracellular vesicles

Introduction

Anthropogenic activities such as mining, smelting, welding and the use of pesticides, fungicides, cosmetics, fuel and certain medication has increased human exposure to heavy metals.[1] Poisonous waste and fumes from mining has led to toxic spills and acid mine drainage.[2] Direct contamination of soil and drinking water through dumping and leakage of material increases the risk of heavy metal exposure especially if this soil and water is used for agricultural purposes as well as drinking and washing.[3]

Heavy metals such as manganese (Mn) and copper (Cu) are essential elements beneficial at low concentrations.[4,5] The metabolism of these metals are tightly regulated, as at high concentrations depending on the route of exposure, these metals may adversely affect the structure and function of the liver, gastrointestinal tract (GIT), kidneys, brain, heart, blood, skin and eyes.[6,7] In contrast, heavy metals such as mercury (Hg), with no beneficial effects, are toxic at low concentrations. Therefore, the established World Health Organisation (WHO) limits for oral exposure is 2.0 mg/L for Cu, 0.5 mg/L Mn and 0.006 mg/L for Hg.[8]

In general, heavy metals are found to promote the production of reactive oxygen species (ROS), bind thiol groups, inhibit enzymes and deplete antioxidant elements such as glutathione (GSH) as well as inhibit antioxidant enzyme activity leading to oxidative stress. This then causes macromolecular damage as well as organelle, cellular and tissue dysfunction.[9] In a previous study done by our research group, we reported that Cu, alone and in combination with Mn and/or Hg, induced hydroxyl radical formation and reduced GSH levels.[10]

Following oral exposure, heavy metals are absorbed by the GIT, then via the blood stream are transported to the liver where metabolism and/or conjugation occurs before excretion by the kidneys.[11,12] Consequently, the liver is a major target of toxicity due to its role in the detoxification process. Chronic heptic injury can result in myofibroblast activation, dysregulated fibrosis and cirrhosis while in addition, continuous cell turnover can lead to the development of hepatocellular carcinoma.[13] This is a major health risk especially for mine workers and those living in mining areas.

Several studies have found that chronic exposure to Cu, Mn or Hg had a severe effect on the liver of rats and mice.[14-20] A major limitation, is that metal concentrations used are based on previously reported dosages and although often high, these studies do identify specific cellular and tissue targets. No studies have investigated the effects of Cu, Mn and Hg as part of mixtures at environmentally relevant concentrations. The duration of exposure and the mode of administration is important and should be relevant. Therefore, the aim of this study, in a subacute model of toxicity[21,22], was to investigate the effects of Cu, Mn and Hg alone and in

combination on the liver tissue of male Sprague-Dawley rats exposed orally to 100 times the WHO acceptable water limits of each metal.

Materials and methods

Sprague-Dawley rat model

The study was conducted using 7-12 week-old, male Sprague-Dawley rats that were obtained from the University of Pretoria Biomedical Research Centre (UPBRC). The rats were kept in a room with a temperature of 22^{o}C (± 2^{o}C), a relative humidity of 50% (± 20%) and a light/dark cycle of 12 hours. This study involved a total of 48 rats. The rats were divided into 8 groups, each group containing 6 rats. Before initiation of oral gavage, the rats were acclimatized for 7 days and the study was then carried out for 28 days. Approval was obtained from the Animal ethics committee of the University of Pretoria (number: h018-17).

Administration of metals

The human dosage of 100 times the WHO water limits for consumption for a 60 kg individual was calculated. The rat equivalent dosage was calculated using the conversion method of Regan-Shaw et al[23]. Each rat received daily 5 ml of the metals daily via oral gavage, 19.4 mM, 5.87 mM and 0.16 µM of Cu (copper (III) sulphate pentahydrate), Mn (manganese (II) chloride) and Hg (mercury (II) chloride) respectively, dissolved in double distilled water. All metal salts were purchased from Sigma Aldrich, Modderfontein, South Africa. Combinations were Cu+Mn, Cu+Hg and Mn+Hg and Cu+Mn+Hg, and the final concentrations in 5ml was the same as for single metals.

Termination

Following standard protocol by the UPBRC, the rats were terminated by an overdose of Isoflurane. Liver tissue was harvested and processed for light microscopy and transmission electron microscopy (TEM).

Light microscopy

The liver tissue was fixed in 2.5% glutaraldehyde (GA)/formaldehyde (FA) and sent to the Department of Paraclinical Sciences in the section of Pathology at the faculty of Veterinary Sciences of the University of Pretoria for standard histological processing. The slides were then stained with haematoxylin and eosin (H&E) and Picrosirius red.[24] The sections were

viewed using a Zeiss AXIO Imager.M2 light microscope (Carl Zeiss Microscopy, Munich, Germany).

Transmission electron microscopy tissue processing

The liver samples were cut into 1 mm^3 size pieces and fixed in 2.5% GA/FA for 1 hour. The samples were then washed three times in a 0.075 M phosphate buffer, pH 7.4, for 15 minutes each. The samples were then placed for 1 hour in the secondary fixative, 1% osmium tetroxide (OsO_4) solution. The samples were then rinsed again and then dehydrated in a series of increasing concentration of ethanol (EtOH); 30%, 50%, 70%, 90% and three changes of 100% EtOH, for 15 minutes each. The samples were embedded in epoxy resin and placed in the oven (60^oC) for 38 hours to harden. Using an ultramicrotome with a diamond knife, 70- 100nm thin sections were cut. The sections were contrasted with uranyl acetate and lead citrate. The sections were viewed using a JEOL JEM 2100F TEM (JEOL Ltd., Tokyo, Japan).

Results

The general morphology of the liver tissue for the control and the single, double and triple metal exposed groups (Fig. 1 and 2) was evaluated. In the control group (Fig. 1 A and B), the typical arrangement of hepatocytes with no hydropic swelling was observed. None to minimal sinusoidal dilation with the presence of erythrocytes and hydropic swelling (black arrows) of hepatocytes was observed in all the metal exposed groups, (Fig. 1 C – P). Binucleated hepatocytes (Bi) were also observed in the exposed groups, particularly in the area surrounding the central vein. Figure 2 illustrates the general structure of the portal triad consisting of a branch of the portal vein (PV), a branch of the hepatic artery (HA) and bile ductules (B), in loose stromal connective tissue. In contrast to the control (Fig 2 A), accumulation of inflammatory cells (white arrows) in the exposed groups (Fig 2 B – H), were observed.

Collagen accumulation within this region was evaluated with Picrosirius red staining. In the control group (Fig. 3 A and B), the collagen of the fibrous stroma surrounding the portal triad, stained red (Fig. 3 A) and with polarized light, green birefringence was observed indicating the presence of thin collagen type III in the stroma (Fig. 3 B). In the exposed groups (Fig 3 C – P), an increase of denser collagen with orange/red birefringence was observed (Fig. 3 D, F, H, J, L, N and P) localized to a small area surrounding the portal triad, with no staining of the surrounding parenchyma. The combinations with the most orange/red birefringence were the

double combinations of Cu + Hg (Fig. 3 K and L) as well as Mn + Hg (Fig. 3 M and N) and the triple combination (Fig. 3 O and P).

To further evaluate effects on the hepatocytes of the liver parenchyma, TEM studies were undertaken. Characteristic features of hepatocytes included centrally located nuclei (N), many mitochondria (M), a prominent rough endoplasmic reticulum (R) and several glycogen granules (G) (Fig. 4). At this magnification no changes were observed when comparing the control (Fig. 4 A) with the different exposure groups (Fig. 4 B – H).

At a higher magnification, damage to the mitochondria was observed and associated structural changes were then evaluated. In the control group (Fig. 5 A and B), the mitochondria (M) appeared normal, with typical shape, minor inner membrane swelling and no membrane damage. In the Cu (Fig. 5 C and D) and Hg (Fig. 5 G and H) exposed groups, the mitochondria appeared normal, with minor inner membrane swelling (white arrow). Prominent inner membrane swelling (white arrow) and membrane damage (blue arrow) was observed in the Mn (Fig. 5 E and F), Cu + Mn (Fig. 5 I and J) and Mn + Hg (Fig. 5 M and N) exposed group with an increase in the inner membrane space. For the Cu + Hg (Fig. 5 K and L) and the triple combination (Fig. 5 O and P) groups inner membrane swelling was also observed, but not to the same extent as was seen in the groups exposed to Mn, alone and in combination. No plasma membrane damage was observed although in all exposed groups extracellular vesicles (EV) were present (Fig. 6 B - H).

Discussion

In a recent study by van Rensburg et al[10] it was reported that at concentrations several fold of the WHO limits for water, Cu alone and together with Mn and Hg as part of a mixture acts as a catalyst of the Fenton reaction and depletes GSH levels due to GSH binding. However, for example in an *in vivo* environment tight regulation of Cu uptake, may reduce ROS associated toxicity. Therefore, in the present study the effects of Cu, Mn and Hg on the liver tissue of exposed Sprague-Dawley rats was investigated using light microscopy and TEM.

In all groups exposed to Cu, Mn or Hg, alone or in combination, minor morphological changes were observed and these were sinusoidal dilation and hydropic swelling. Hydropic swelling or hydropic degeneration has been identified as the first stage of liver injury and is characterized by the vacuolization and cellular swelling.[25] This is a reversible change that is most often caused by water and sodium accumulation due to membrane disturbances that reduces the ability of a cell to maintain fluid and ionic homeostasis.[25] These features are consistent with cells undergoing oncosis, a pre-lethal phase following cell injury due to subsequent exposure

to drugs or chemicals.[26] Characteristics of oncosis include cellular and organelle swelling, dilation of the endoplasmic reticulum and Golgi apparatus, mitochondrial and chromatin condensation and formation of cytoplasmic blebs or blisters which is associated energy depletion which interferes with the membrane ion pumps.[27,28]

Numerous studies that have investigated the effects of Cu, Mn or Hg individually, have found that these metals caused necrosis with characteristic pyknotic nuclei and hyper-eosinophilic cytoplasm.[15-19] In the present study, no pyknotic nuclei or hepatocytes with hyper-eosinophilic cytoplasm were observed in the metal exposed groups. This may be due to the lower concentrations and exposure times used compared with previous studies investigating the toxicity of metal combinations.[29]

Combinations, Cu+Hg and Mn+Hg appeared to be the most toxic compared with Cu+Mn, and these combinations caused a greater degree of hydropic swelling and sinusoidal dilation. This indicates that type of metals rather than concentrations determines the extent of toxicity with possible synergistic interactions between Cu, Mn and Hg.

Another feature that was observed in all the exposed groups were the presence of binucleated hepatocytes (Bi) mostly prominent around the central vein (CV), a feature that has been associated with hepatotoxicity.[30,31] Recent studies have found that polyploidization is an adaption mechanism for stress, particularly following xenobiotic, chemical and oxidative damage,[20] although other studies have identified it to be a sign of regeneration.[18]

In this study, the presence of fibrosis was evaluated following Picrosirius red staining used to visualize type I and type III collagen fibers as well as muscle fibers.[24] In the portal triad there is usually an equal proportion of type I and III collagen but with fibrosis and cirrhosis, type I collagen is more prominent.[32] Collagen was observed surrounding the portal triad where the control group exhibited a larger proportion of type III collagen while in all the exposed groups, type I collagen was more prominent, a characteristic feature of early liver fibrosis. Following absorption, metals are transported via the vascular system to the liver, blood from the portal vein and artery drains into the central vein. This ensures the optimal presentation of the blood to the hepatocytes and cells of the innate immune system.[11] When entering the liver tissue, metal levels in the portal triad are high, and subsequent accumulation and activation of the cells of the innate immune system can occur. Subsequent reported effects, are the differentiation of HSC into myofibroblasts and subsequent accumulation of collagen type I in this region.[33,34]

Further detailed evaluation of organelle structure revealed mitochondrial damage such as membrane damage and inner membrane swelling especially for Mn and metal combinations.

Chronic exposure to Mn results in the accumulation of Mn in hepatic mitochondria.[35,36] Manganese acts as a co-factor of several mitochondrial enzymes and these includes the antioxidant enzyme, superoxide dismutase (SOD), as well as xanthine oxidase and pyruvate carboxylase.[41] Consequently, disruption of SOD and the activity of other enzymes can lead to the disruption of biochemical pathways and leading to oxidative stress, organelle damage and subsequent cellular dysfunction.

Extracellular vesicles were identified in the metal exposed groups. The formation of EV, including microvesicles (MV) is associated with liver disease and mediated intracellular communication between hepatocytes activates several processes such as monocyte/macrophage chemotaxis and activation as well as the migration and activation of HSC.[44] Hirsova and Gores[37], described a pro-inflammatory feed-forward loop between lipotoxic hepatocytes and activated macrophages. Lipotoxicity induces the release of hepatic extracellular vesicles which are then engulfed by resident and recruited macrophages inducing macrophage activation. This leads to increased expression of death receptor ligands (Fas ligand), TNF-related apoptosis inducing ligand (TRAIL) and pro-inflammatory cytokines such as tumour necrosis factor (TNF-∝) which induces hepatocyte apoptosis and furthers inflammation.[37] The mitochondria was identified as the initial site of palmitic acid, lipotoxicity and exposure led to mitochondrial ROS generation and upregulation of TNF-∝.[38] Hirsova et al[39] identified that extracellular vesicles in non-alcoholic steatohepatitis (NASH) mediated monocyte/macrophage chemotaxis to the liver, while TRAIL-enriched extracellular vesicles contributed to macrophage activation leading to fibrosis.

Both Cu and Mg, but not Hg, are catalysts of the Fenton reaction while both Mn and Hg, but not Cu binds GSH[10]. Furthermore the disruption of SOD by Mn, can further reduce cellular antioxidant capacity. Subsequent development of oxidative stress, that compromises mitochondrial function can potentially cause macrophage activation, leading to fibrosis via a pro-inflammatory feed-forward loop as described by Hirsova and Gores[37] for lipotoxicity.

The extent of damage following exposure of rats to 100x the WHO limits for water for 28 days indicates an inflammatory response and extracellular vesicle formation. Especially Hg combined with Mn and Cu in double and triple combinations causes fibrosis at the portal triad and while Mn, combined with Cu and Hg as double and a triple combination compromises mitochondrial structure. This study, identifies that these metals alone and in combination are toxic. Although the mechanism of specific metals and combinations may differ all metals as double and triple combination, in varying degrees contributes to fibrosis in the portal triad and altered mitochondrial functioning in hepatocytes.

Conclusion

Based on observations of accumulation of inflammatory cells and fibrosis at the portal triad, mitochondrial swelling and membrane damage as well as the formation of EV, a ROS mediated process of toxicity may occur following exposure to metals Cu, Mn and Hg.

Recommendations

Future studies should focus on the mechanisms involved and could include identifying intracellular sites of metal accumulation, the intracellular mechanism of ROS generation and subsequent effects on mitochondria function, leading to inflammation, fibrosis development increasing the risk of associated disease.

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Disclosure statement

The authors declare no conflict of interest.

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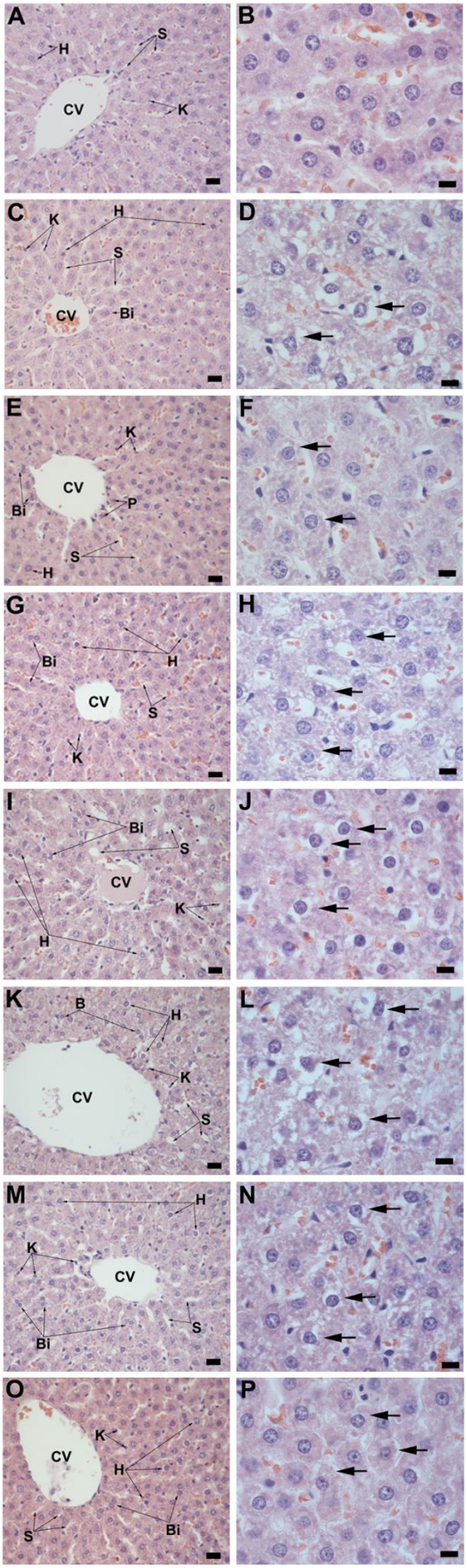
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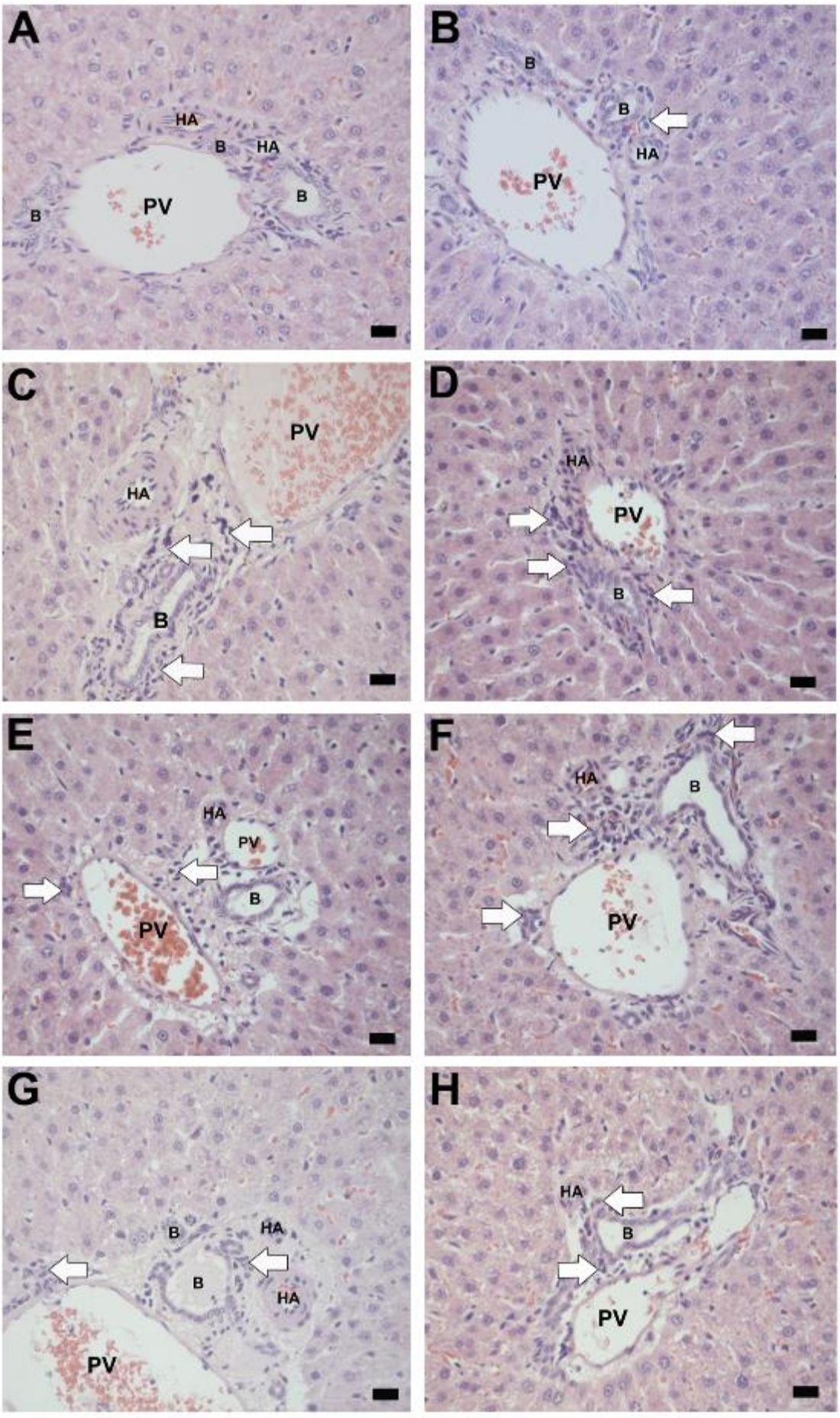
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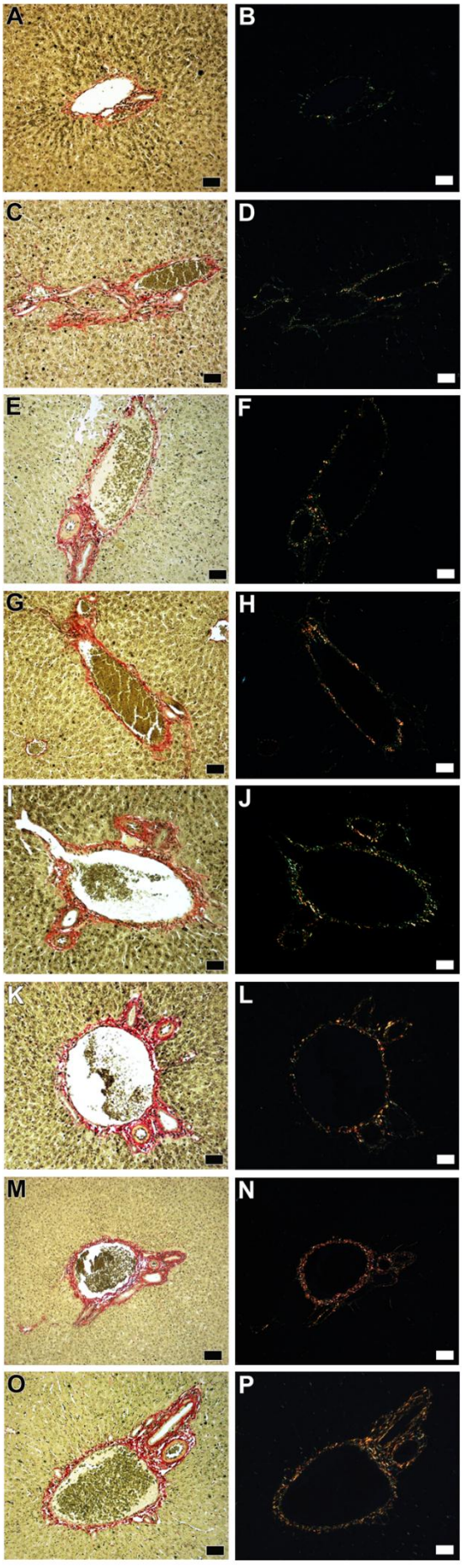
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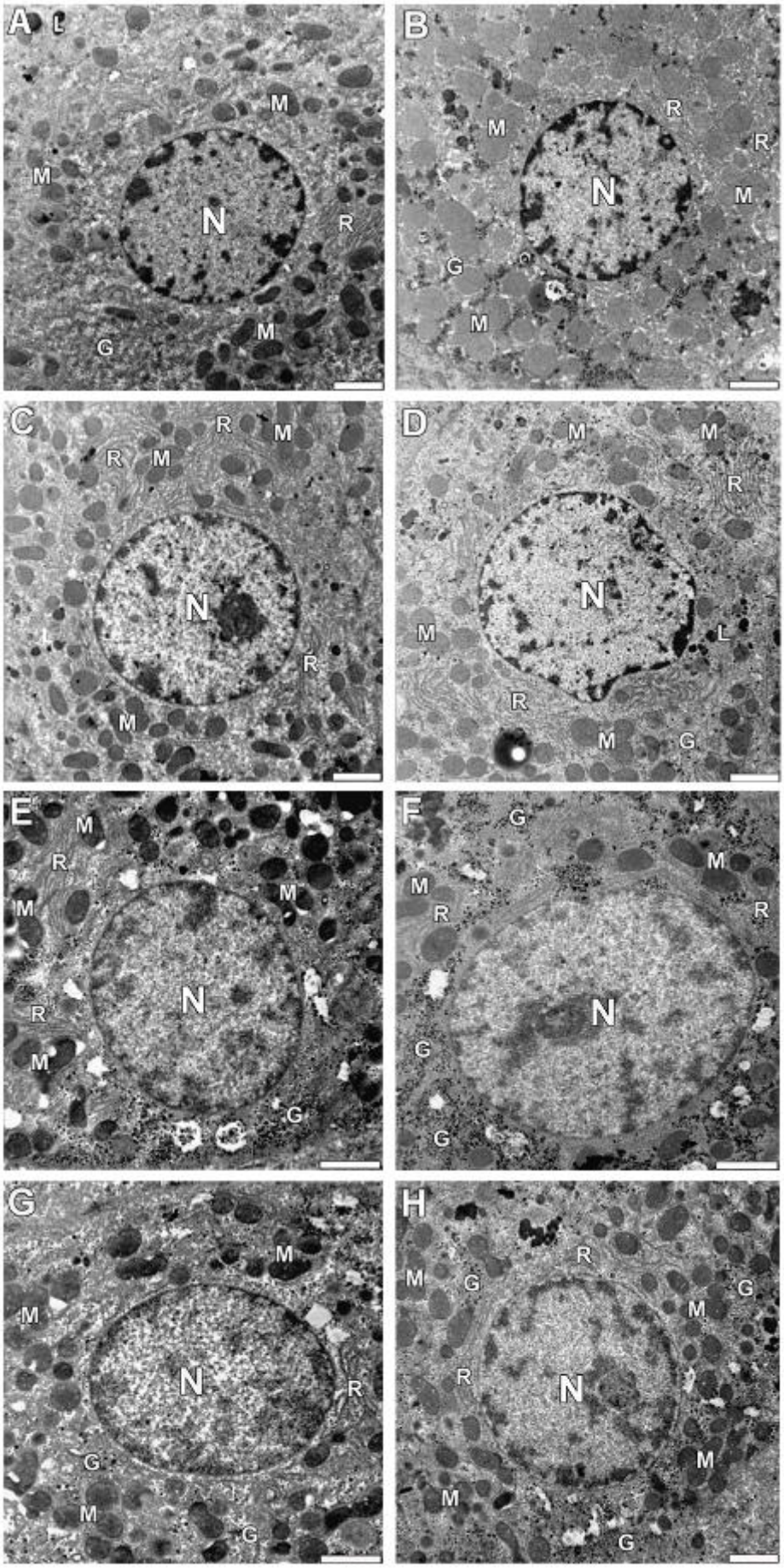
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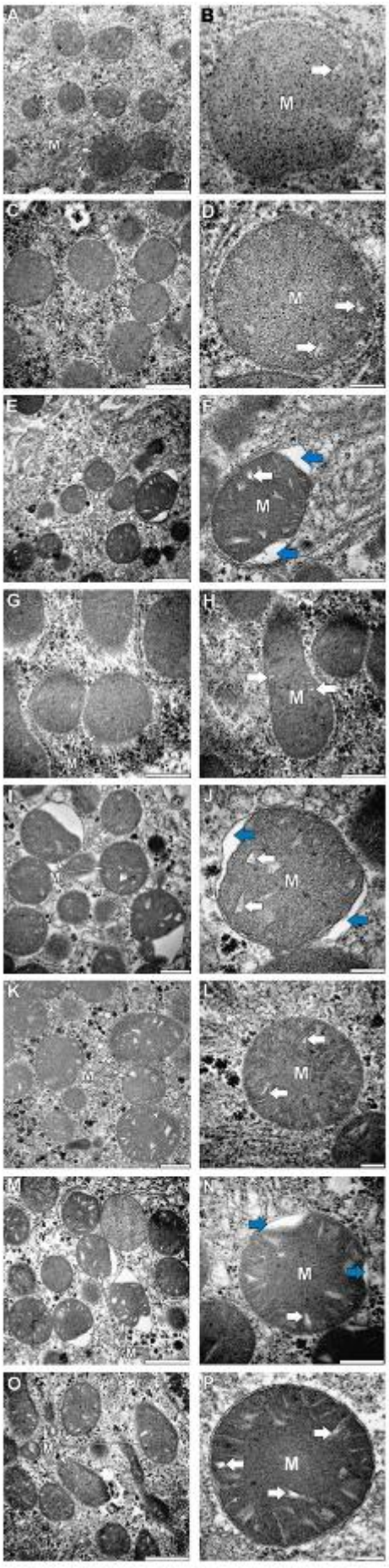
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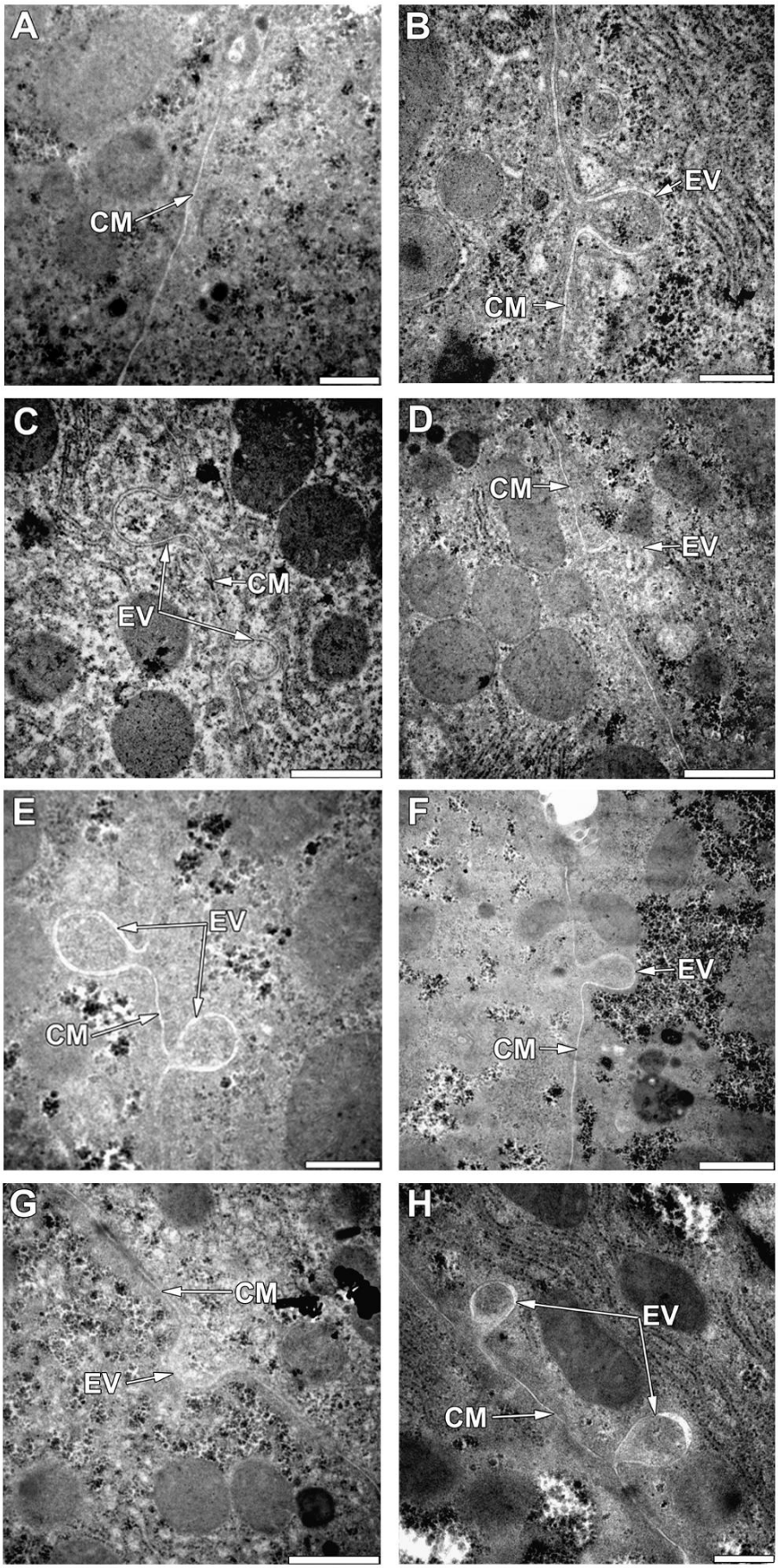
N
M
O
M
P
M


A
CM
B
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CM
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G
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EV
CM

Figure legends

Figure 1: Light microscopy micrographs of liver tissue illustrating the central vein, hepatocytes and sinusoid arrangement of the control (A and B), Cu (C and D), Mn (E and F), Hg (G and H), Cu and Mn (I and J), Cu and Hg (K and L), Mn and Hg (M and N) and triple combination (O and P) groups. **Stain:** H&E. **Key:** Bi: Binucleation, CV: Central vein, H: Hepatocytes, K: Kupffer cells and S: Sinusoids. Black arrows indicate hydropic swelling of the hepatocytes. (Scale bars: A, C, E, G, I, K, M and O: 20 µm; B, D, F, H, J, L, N and P: 10 µm).

Figure 2: Light microscopy micrographs of liver tissue illustrating a portal triad of the control (A), Cu (B), Mn (C), Hg (D), Cu and Mn (E), Cu and Hg (F), Mn and Hg (G) and triple combination (H) groups. **Stain:** H&E. **Key**: B: Bile ductules, HA: Branch of hepatic artery and PV: Branch of portal vein. White arrows indicate an accumulation of inflammatory cells. (Scale bar: 20 µm).

Figure 3: Light microscopy micrographs of liver tissue illustrating a portal triad of the control (A and B), Cu (C and D), Mn (E and F), Hg (G and H), Cu and Mn (I and J), Cu and Hg (K and L), Mn and Hg (M and N) and triple combination (O and P) groups. **Stain:** Picrosirius Red. Figures A, C, E, G, I, K, M and O are bright field micrographs and figures B, D, F, H, J, L, N and P are polarized light micrographs. (Scale bars: 50 µm).

Figure 4: Transmission electron micrographs of the hepatocytes of liver tissue of the control (A), Cu (B), Mn (C), Hg (D), Cu and Mn (E), Cu and Hg (F), Mn and Hg (G) and triple combination (H). **Key**: G: Glycogen granules, L: Lysosomes, N: Nucleus, M: Mitochondria and R: Rough endoplasmic reticulum. (Scale bars: 2 µm).

Figure 5: Transmission electron micrographs of the mitochondria of the liver tissue of the control (A and B), Cu (C and D), Mn (E and F), Hg (G and H), Cu and Mn (I and J), Cu and Hg (K and L), Mn and Hg (M and N) and triple combination (O and P) groups. Figures A, C, E, G, I, K, M and O illustrate mitochondria, and figures B, D, F, H, J, L, N and P illustrate a single mitochondrion at higher magnification. **Key**: M: Mitochondria. White arrows indicate inner membrane swelling and blue arrows indicate membrane damage. (Scale bars: A, C, E, M and O: 1 µm; F, G, I, K, N: 500 nm; B, D, H, J, L and P: 200 nm).

Figure 6: Transmission electron micrographs of the cell membranes of the liver tissue of the control (A), Cu (B), Mn (C), Hg (D), Cu and Mn (E), Cu and Hg (F), Mn and Hg (G) and triple combination (H) groups. The cell membrane of the hepatocytes as well as extracellular vesicles are shown. **Key**: CM: Cell membrane and EV: extracellular vesicles. (Scale bars: C, D, F and G: 1 µm; A, B, E and H: 500 nm).