Histological analysis of the effects of cadmium, chromium and mercury alone and in combination on the spleen of male Sprague-Dawley rats

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Abstract

The mining sector in South Africa is expected to be the fifth largest in the world. Both mining and transport are the most common reasons for an increased risk of human exposure to heavy metal contamination in South Africa. Due to increasing amounts of metals in the environment this study identified three metals cadmium, chromium and mercury based on the risk of exposure in South Africa. The aim of this study was to investigate the changes in the morphology of the spleen tissue of male Sprague-Dawley rats exposed to these metals alone and in combination by using light microscopy. Forty eight animals in eight experimental groups were exposed, via oral gavage, to these metals at 1000x the World Health Organization's acceptable water limits of each respective metal, alone and in combination, for 28 days. Changes in the histological structure of the spleen were observed using haematoxylin and eosin and picrosirius red staining. Necrosis was observed in all the groups, with the severity varying between the different exposure groups, alone or in combination. Fibrosis in the spleen tissue was only seen in the experimental groups exposed to cadmium and mercury respectively, as well as in the combination of cadmium and mercury.

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KEYWORDS: Heavy metals, toxicity, spleen, light microscopy, morphology, fibrosis.

Introduction

The term heavy metal refers to any metallic chemical element that has a relatively high density and is poisonous or toxic at low concentrations. Examples of heavy metals include cadmium (Cd), chromium (Cr), mercury (Hg), arsenic (As), thallium (Tl), and lead (Pb), to mention a few.[1] Heavy metals are natural elements of the earth's crust. They originate spontaneously and cannot be degraded or destroyed. To a small extent, heavy metals can enter the body via either air, food and/or drinking water. Some heavy metals are needed to maintain metabolism of the human body, however, at higher concentrations these metals can be poisonous.[2] The two most common sources of heavy metal pollution in the environment are either natural or anthropogenic. The leading anthropogenic sources of human exposure to heavy metals include agriculture, metallurgy, transport and mining. The activities mentioned are also the leading sources of increased environmental heavy metal exposure.[3] The mining sector in South Africa is expected to be the fifth largest in the world.[4] Both mining and transport are the most common reasons for an increased risk of human exposure to heavy metal contamination in South Africa. Communities residing in regions that are contaminated are the most vulnerable, specifically children and pregnant women. Heavy metals are inhaled, ingested and/or absorbed through the skin.[4] The vulnerability of these communities is because they generally use contaminated water for drinking, the preparation of food, the irrigation of crops and bathing.

The extent of heavy metal toxicity is greatly dependent on duration, dosage, route of administration as well as other physiological functions.[4] Several studies confirm that there is an increase in the levels of metals, specifically Cr, zinc, Pb, manganese, titanium, strontium, tin and copper in a variety of water sources. This results in simultaneous contamination from different heavy metals. Therefore individuals are generally at risk of a combination of metals at varying concentrations.[5] In this study, three heavy metals, Cd, Cr and Hg, were identified based on the risk of exposure in South Africa. In addition, it was previously found that all three of these metals have a high bio-accumulation in the spleen, with Cr having the highest bio-accumulation of the three.[1] Since the spleen plays an important supporting role in the body, it is an important organ to keep in mind when investigating heavy metal toxicity. The effects of these metals on the spleen were evaluated to determine the possible toxic effect of Cd, Cr and Hg, alone and in combination, on the spleen.

Materials and methods

Sprague-Dawley Rat Model

Six week old male Sprague-Dawley rats (200-250 g) were obtained from the University of Pretoria Biomedical Research Centre (UPBRC). The animals were housed in conventional cages complying with the sizes laid down in the 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. All experimental protocols complied with the requirements of the University of Pretoria Animal Ethics Committee (Animal ethics numbers: H007-15 and H009-15). Forty eight rats were randomly divided into 8 groups, containing 6 rats each. The animals were allowed to acclimatise for 7 days prior to exposure for 28 days.

Metal Administration

Cadmium chloride ($CdCl_2$), potassium dichromate ($K_2Cr_2O_7$) and mercury chloride ($HgCl_2$) [Merck (Pty) Ltd, South Africa] were dissolved in sterile water and was administered to the rats via oral gavage. The control rats only received saline. The rats were exposed to the metals as indicated in Table 1. These concentrations where chosen based on the World Health Organization (WHO) acceptable water limits (mg/L) for Cd, Cr and Hg times 1000.[6] The metal dosage (mg/L) for an average person of 60.7 kg, drinking 1.4 L of water per day was calculated and converted using the dose equation of Reagan-Shaw et al.[7] Rats were weighed daily to monitor any weight loss during the experimental period and this data was statistically analysed. The dosages were adjusted weekly according to average weekly weight of the rats. The dosage and duration of exposure represents chronic metal exposure in humans.

Termination

The rats were terminated via isoflurane overdose, according to standard methods employed by the UPBRC. The spleen was harvested, cut into 5-10mm^3 blocks and processed for light microscopy. Blood was collected in heparin tubes to measure the plasma levels of Cd, Cr and Hg in all of the experimental groups.

Light Microscopy

The spleen tissue was fixed in 2.5% glutaraldehyde (GA)/formaldehyde (FA) for an hour, rinsed three times in 0.075 M sodium phosphate (Na_3PO_4) buffer (pH 7.4) for 15 minutes each before the tissue samples was dehydrated in 30%, 50%, 70%, 90% and three changes of 100% ethanol. The tissue samples were then left overnight in 100% ethanol. The next day the spleen samples were placed in solution of 50% xylene and 50% 100% ethanol for 30 minutes, where after it was placed in xylene for 2 hours. The samples were then placed in 30% paraffin wax

(Sigma-Aldrich, South Africa) and 70% xylene solution for an hour at 60ºC, followed by another hour in 70% paraffin wax and 30% xylene solution at 60ºC. Finally, the samples were placed in 100% paraffin wax for 2 hours at 60ºC. The spleen samples were then placed in a steel mould, filled with paraffin wax and a marked grid was placed on top. The moulds with the marked grids were placed on a cooling plate to allow the wax to cool and harden. Sections of 3-5 µm were made with a Leica RM 2255 wax microtome (Leica Microsystems, Wetzlar, Germany). Tissue sections were stained with haematoxylin and eosin (H&E) to evaluate the general tissue morphology, as well as stained with picrosirius red to investigate possible fibrosis in the tissue using polarized light microscopy. The slides were viewed with a Zeiss AXIO Imager M2 light microscope (Carl Zeiss Microscopy, Munich, Germany).

Statistical analysis

Statistical analysis on the weights of the rats were performed on GraphPad Prism Version 6.01 using 1-way analysis of variance (ANOVA) and Tukey's multiple comparisons test, where a p-value of ≤ 0.05 was considered significant.

Results and discussion

Analysis of weights

The rats were weighed daily during the experimental period and no significant differences were seen between the groups.

Plasma levels of heavy metals

Plasma levels of the heavy metals in the blood of the rats showed that the heavy metals Cd, Cr and Hg were absorbed (Table 2) and consequently organ exposure occurred; it also confirms that the *in vivo* model was successfully implemented. An increase in Cd plasma levels can also

be seen in the Cd and Cr and triple combination groups when compared with the individual metal exposure groups.

General morphology: Haematoxylin and Eosin staining

For light microscopy analysis, the following features associated with cellular damage were evaluated: necrosis (cell swelling, vacuolation, karyolysis and loss of cellular content)[8] and a reduction of cell density. Evaluation of red pulp (Fig. 1 A) and white pulp (Fig. 1 B) in the control group revealed no cellular alterations with morphology typical to that of control samples. In the Cd exposed group (Fig. 1 C and D), minor changes were seen, with minimal decrease in cellular density being observed close to the capsule (Fig. 1 C) and surrounding the central artery (Fig. 1 D). In the Cr exposed group (Fig. 1 E and F) moderate changes are seen in the tissue, with a moderate decrease in cellular density close to the capsule (Fig. 1 E) and surrounding the central artery (Fig. 1 F). In the Hg exposed group (Fig. 1 G and H) severe changes are seen in the red- (Fig. 1 G) and white pulp (Fig. 1 H). All the combination groups (Fig. 1 I – O) showed severe alterations in their cellular morphology with depletion of cellular density being observed in close approximation to the capsule and surrounding the central artery. All groups, except the control group showed indications of necrosis (black arrows). The severity of cellular depletion varied between different experimental groups. A summary of the histological changes observed in the spleen is presented in Table 3.

Fibrosis: Picrosirius red

Evaluation of the capsule (Fig. 3 A and B) and the central artery (Fig. 5 C and D) in the control group revealed moderate amounts of collagen present in the capsule and mild amounts of collagen was seen surrounding the central artery. In the Cd exposed experimental group (Fig. 3 and 5 C and D), a moderate amount of collagen was observed in the capsule (Fig. 3 D) and a

moderate amount of collagen deposition was observed surrounding the central artery (Fig. 5 D). In the Cr exposed experimental group (Fig. 3 and 5 E and F), mild amounts of collagen were observed in the capsule (Fig. 3 F) and no collagen deposition was observed surrounding the central artery (Fig. 5 F). In the Hg exposed experimental group (Fig. 3 and 5 G and H) a moderate amount of collagen was observed in the capsule (Fig. 3 H) and a moderate amount of collagen deposition was observed surrounding the central artery (Fig. 5 H). In the Cd and Cr combination group (Fig. 4 and 6 I and J) a moderate amount of collagen was observed in the capsule (Fig. 4 J) and moderate amounts of collagen deposition was observed surrounding the central artery (Fig. 6 J). In the Cd and Hg combination exposed experimental group (Fig. 4 and 6 K and L) severe amounts of collagen was observed in the capsule, some degree of capsule thickening was also observed (Fig. 4 L). Severe amounts of collagen deposition were also observed surrounding the central artery (Fig. 6 L). The normal round structure of the central artery also appears to be altered. In the Cr and Hg combination group (Fig. 4 and 6 M and N) a moderate amount of collagen was observed in the capsule (Fig. 4 M) and moderate amounts of collagen deposition was observed surrounding the central artery (Fig. 6 N). The normal round structure of the central artery also appears to be altered. In the Cd, Cr and Hg combination exposed experimental group (Fig. 4 and 6 O and P) severe amounts of collagen were observed in the capsule (Fig. 4 P) and severe amounts of collagen deposition was observed surrounding the central artery (Fig. 6 P). The normal round structure of the central artery also appears to be altered. The amount of collagen is directly related to the amount of fibrosis taking place within tissue. A summary of the changes in collagen depositions observed in the spleen is presented in Table 4.

Discussion

South Africa is a country rich in mineral resources and therefore it is estimated to be the fifth largest mining sector in the world. Mining is one of the most common sources of heavy metal pollution and they do not only pose a risk to the environment but can also pose a potential risk to animal and human health. Communities residing in regions that are contaminated with heavy metal pollution are the most vulnerable, specifically children and pregnant women.[4] The vulnerability of these communities is due to the fact that they generally use contaminated water for drinking, the preparation of food, the irrigation of crops and bathing. The extent of heavy metal toxicity is also greatly dependant on duration, dosage, route of administration as well as other physiological functions specific to an organism and several studies confirm that there is an increase in the levels of heavy metals.[4-5] Based on reports on contaminated water in the mining areas of South Africa, three heavy metals Cd, Cr and Hg were chosen.[9] In this study, a Sprague-Dawley rat model was used to investigate the effects of these heavy metals on the spleen. There were no significant changes in the weights between the control and metal-exposed groups, but there was a steady increase in the weights throughout the duration of the study. The presence of all the metals in the blood plasma indicated that the study was implemented successfully and it seems that Cd in combination with the Cr, is processed at a different rate than when administered alone or in combination with Hg.

The effects of Cd, Cr and Hg alone and in combination on the spleen tissue were examined by using light microscopy. During the general light microscopy analysis, the red- and white pulp regions of the tissue were chosen, as they are the regions in the spleen that play a major role in the immune function. Lymphocytes are present in the white pulp region and macrophages are present in the red pulp region.[10] Lymphocytes are a type of white blood cell that functions as part of the body's immune system. There are three main types of lymphocytes: T-cells, B-cells

and NK cells.[10] In the current study, no cellular alterations were observed surrounding the central artery (white pulp region) in the control group (Fig. 1 B). The Cd (Fig. 1 D) and Cr (Fig. 1 F) individually exposed groups showed minimal and moderate necrosis of cells in the white pulp region. The Hg exposed group (Fig. 1 H) revealed severe necrosis of cells in the white pulp region. In a similar study, Ilbäck in 1991[11] found that after exposure to Hg the NK cell numbers were depleted and thus supports the loss in cells that were found in the white pulp in this current study. All the combination groups revealed severe necrosis in the cells located in the white pulp region. In the Cd and Cr combination group (Fig. 2 J) the severity of necrosis may be attributed to the fact that Cd, which causes minimal necrosis alone, and Cr, which causes moderate necrosis by itself, to have an additive effect when administered in combination. To our knowledge, no literature pertaining to the effects of simultaneous exposure to heavy metals on the spleen is available and therefore there were no comparison to previous literature in terms of the metal combination groups.

In the red pulp areas of the spleen, macrophages form an integral part of the immune system and its main function is to locate foreign bodies, engulf and then digest them through the process of phagocytosis.[10] In the spleen it mainly engulfs old and/or damaged red blood cells. No cellular alterations were observed in the red pulp region of the control group (Fig. 1 A). The Cd exposed group (Fig. 1 C) showed minimal depletion of cells in the red pulp region, this is similar to previous literature in which some necrosis was seen as a result of Cd toxicity in the spleen by Dumkova and co-authors in 2016.[12] The Cr exposed group (Fig. 1 E) revealed a moderate depletion of cells in the red pulp region, this occurrence is similar to what was observed during a previous study performed by Das Neves,Santos,de Pereira and de Jesus.[13] It was concluded that Cr toxicity led to the depletion of red pulp cells which is directly related to the process of phagocytosis. The Hg exposed group (Fig. 1 G) revealed severe depletion of cells in the red pulp region. Mercury has a high bio-accumulation within the spleen, specifically

the red pulp region which may lead to an increase of apoptosis of cells being constantly exposed to this metal.[14] All the combination groups revealed severe depletion in the cells located in the red pulp region. The severity of the cellular alterations in the Cd and Cr combination group (Fig. 2 I) may be attributed to an additive effect when administered in combination. Although no studies have been done on the spleen and simultaneous metal exposure, other studies have been done on the effects of heavy metals alone and in combination on the liver and kidney tissue and coagulation system. Some alterations to tissues and cells were found and thus the alterations to the spleen tissue seen in the present study is expected.[15-16]

During the analysis of possible fibrosis in the spleen the capsule and central artery were chosen for analysis as they lie in close approximation to the red and white pulp respectively which represents the major areas playing an important role in the function of the spleen. Evaluation of the control group revealed moderate amounts of collagen in the capsule and mild amounts of collagen are seen surrounding the central artery. This is similar to the literature, since the capsule is made of dense fibrous tissue and moderate amounts of collagen is expected.[10] Irregularly spaced trabeculae and central arteries surrounded with smooth muscle and fibro-elastic tissue are also present within the spleen.[17] The Cr exposed experimental group showed no difference in the amount of collagen present when compared to the control. Moderate increases in the amounts of collagen were observed in the Cd (Fig. 3 and 5 C and D), Hg (Fig. 3 and 5 G and H), Cd and Cr (Fig. 4 and 6 I and J) and Cr and Hg (Fig. 4 and 6 M and N) exposed experimental groups. Since no fibrosis was observed in the Cr individually exposed experimental group, the increase in presence of collagen in the combination groups containing Cr (Cd and Cr as well as Cr and Hg) may be attributed to the individual effects of Cd and Hg to induce fibrosis. The Cd and Hg (Fig. 4 and 6 K and L) and the Cd, Cr and Hg (Fig. 4 and 6 O and P) combination groups both revealed severe fibrosis. The increase in the severity of the

fibrosis may be attributed to an additive effect of Cd and Hg in combination. Fibrosis within the spleen is mainly as a result of injury, inflammation or infarction.[18] According to this study, Cd and Hg are mainly responsible for toxicity leading to fibrosis within the spleen, whereas Cr exposure resulted in none or minimal alteration, similar to what was found in the control group. The capsule was more affected in comparison to that of the central artery, this was also seen in previous studies, as capsular fibrosis is more common within the spleen and it will typically occur as a localized lesion, which is similar to what was observed during this study. Parenchymal fibrosis can also surround or infiltrate peri-arteriolar lymphoid sheaths.[18] A summary of the observed changes are shown in Tables 3 and 4.

Conclusion

In conclusion, Cd, Cr and Hg alone and in combination causes necrosis at different degrees depending on the metal being examined as well as if they were administered alone or in combination. The majority of combination groups showed more extensive cellular alterations and this was concluded to be as a result of the additive effects these metals have when administered in combination. The degree of necrosis was more severe in the Hg exposed group and all of the combination groups compared to the control. Cd and Hg alone and in combination cause fibrosis in spleen tissue, but the Cr group was similar to that of the control indicating no or minimal fibrosis. Fibrosis took place predominantly in the capsule but some degree of collagen deposition is also observed within the tissue. This study concluded that the heavy metals Cd, Cr and Hg cause alterations in the cellular morphology of the spleen. These alterations may lead to a malfunctioning of the spleen, which is an important organ of the immune system. Compromising the immune system may leave individuals vulnerable to other opportunistic diseases.

Acknowledgements

The authors would like to thank Ms Ilse Janse van Renburg and other staff members at the University of Pretoria Biomedical Research Centre for their professional help during the animal study.

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TABLES

Table 1: ***In vivo*** **control and metal dosages**

Groups	Dosages	Days
Control	0.5 mL Saline	28
Cd	0.854 mg/kg body weight	28
Cr	14.22 mg/kg body weight	28
Hg	1 mg/kg body weight	28
Cd and Cr	0.854 mg/ kg and 14.22 mg/kg body weight	28
Cd and Hg	0.854 mg/kg and 1 mg/kg body weight	28
Cr and Hg	14.22 mg/kg and 1 mg/kg body weight	28
Cd, Cr and Hg	0.854 mg/kg and 14.22 mg/kg and 1 mg/kg body weight	28

Table 2: Administered dosages and blood levels of metals

Group	Administered dose (µg/kg)	Plasma concentrations (µg/ℓ)	Plasma concentrations (µM)
		Mean ± SD	**Mean ± SD**
Cd	696.01	1.872 ± 0.37	0.010 ± 0.002
Cr	20619.00	1054.52 ± 864.59	3.55 ± 2.91
Hg	1147.50	35.41 ± 6.37	1.30 ± 0.23
Cd+Cr	696.01 and 20619.00	3.6 ± 0.82 and 1015.46 ± 513.97	0.020 ± 0.0045 and 3.42 ± 1.73
Cd+Hg	696.01 and 1147.50	2.60 ± 0.42 and 33.20 ± 8.73	0.014 ± 0.002 and 1.22 ± 0.32
Cr+Hg	20619.00 and 1147.50	759.90 ± 251.77 and 32.34 ± 4.90	2.56 ± 0.85 and 1.19 ± 0.18
Cd+Cr+Hg	696.01, 20619.00 and 1147.50	3.73 ± 0.94, 1080.00 ± 580.25 and 28.77 ± 5.84	0.020 ± 0.005, 3.63 ± 1.95 and 1.06 ± 0.22

SD: standard deviation

Table 3: Summary of the histological changes in the spleen tissue

Experimental group	Necrosis	Decrease in cellular density
Control	-	-
Cd	+	+
Cr	++	++
Hg	+++	+++
Cd and Cr	+++	+++
Cd and Hg	+++	+++
Cr and Hg	+++	+++
Cd, Cr and Hg	+++	+++

-, none; +, minimal; ++, moderate; +++, severe

Table 4: Summary of the changes in collagen deposition observed in the spleen

Experimental group	Capsule	Central artery
Control	+	+
Cd	++	++
Cr	+	-
Hg	++	++
Cd and Cr	++	++
Cd and Hg	+++	+++
Cr and Hg	++	++
Cd, Cr and Hg	+++	+++

-, none; +, minimal; ++, moderate; +++, severe

FIGURES

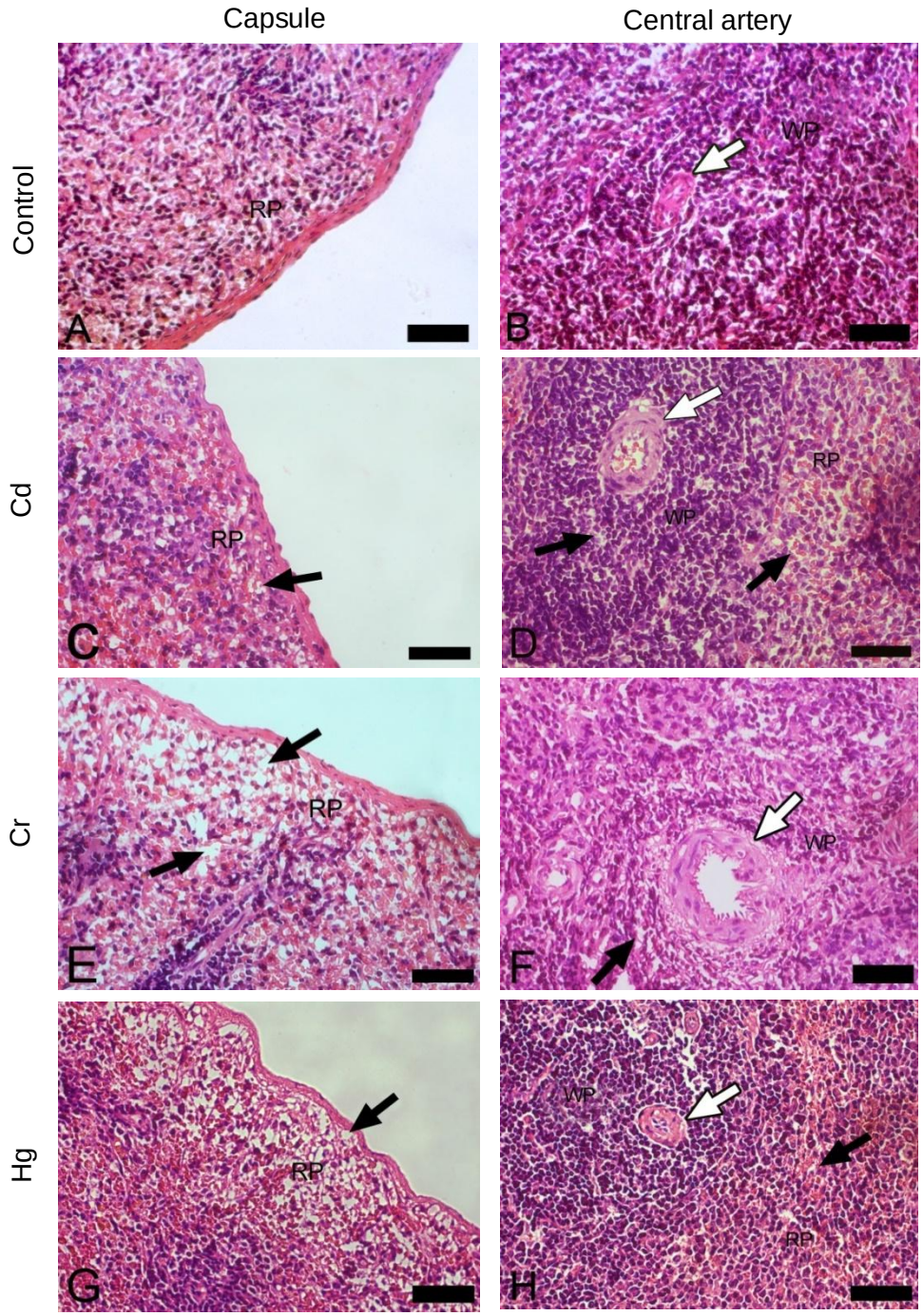

Fig. 1

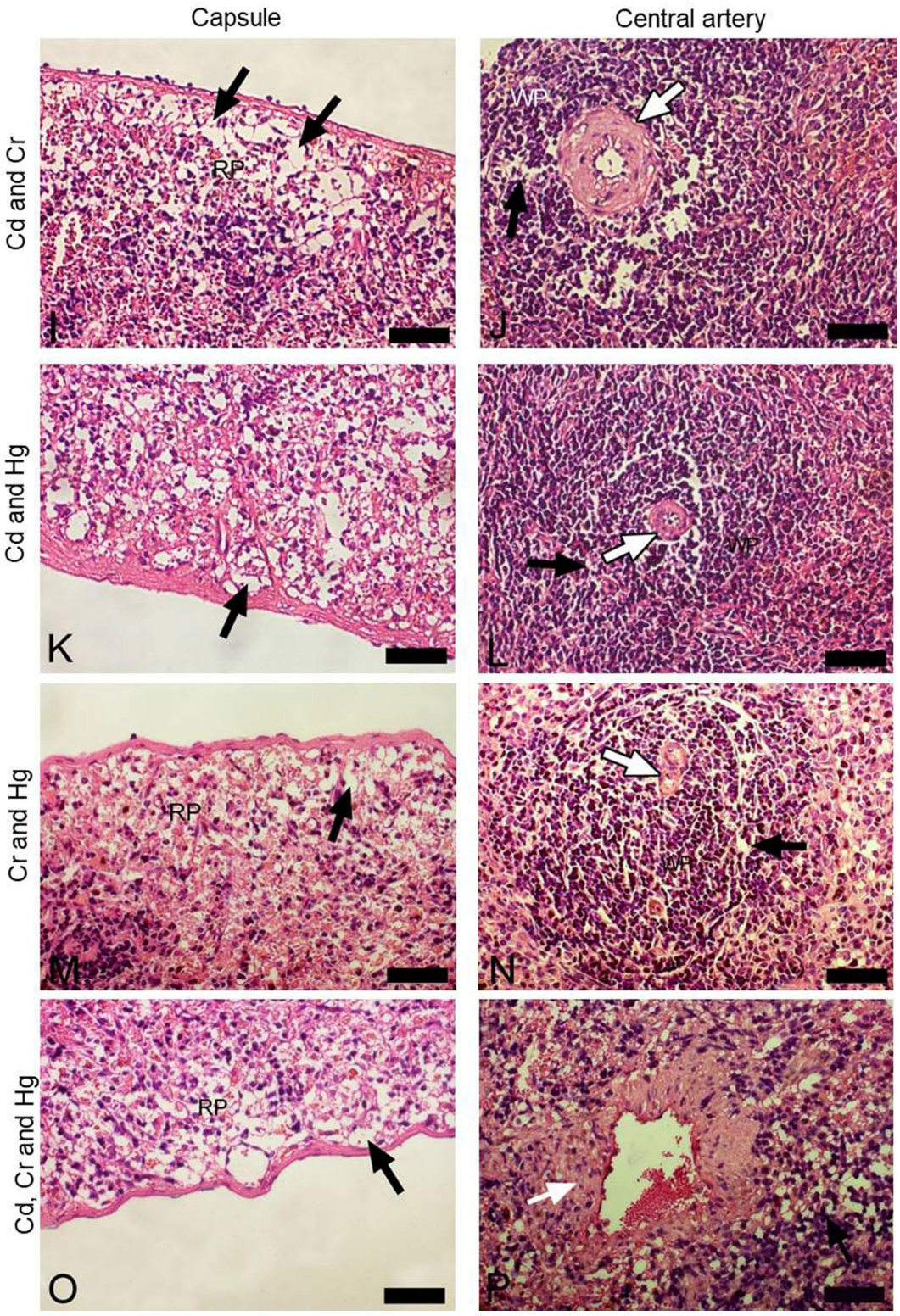
Capsule
Central artery
Cd and Cr
Cd and Hg
Cr and Hg
Cd, Cr and Hg
RP
VP
I
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Fig. 2

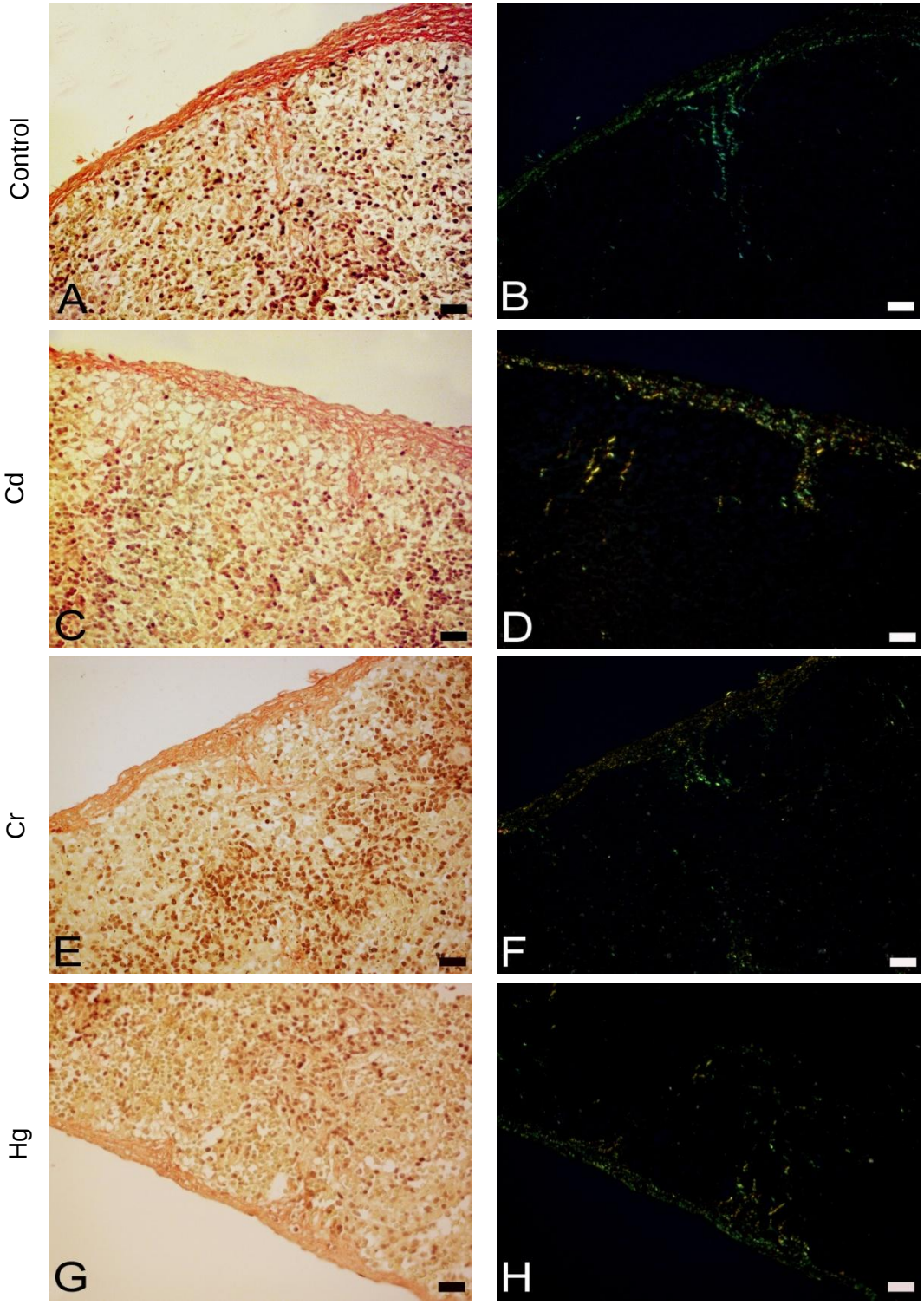

Fig. 3

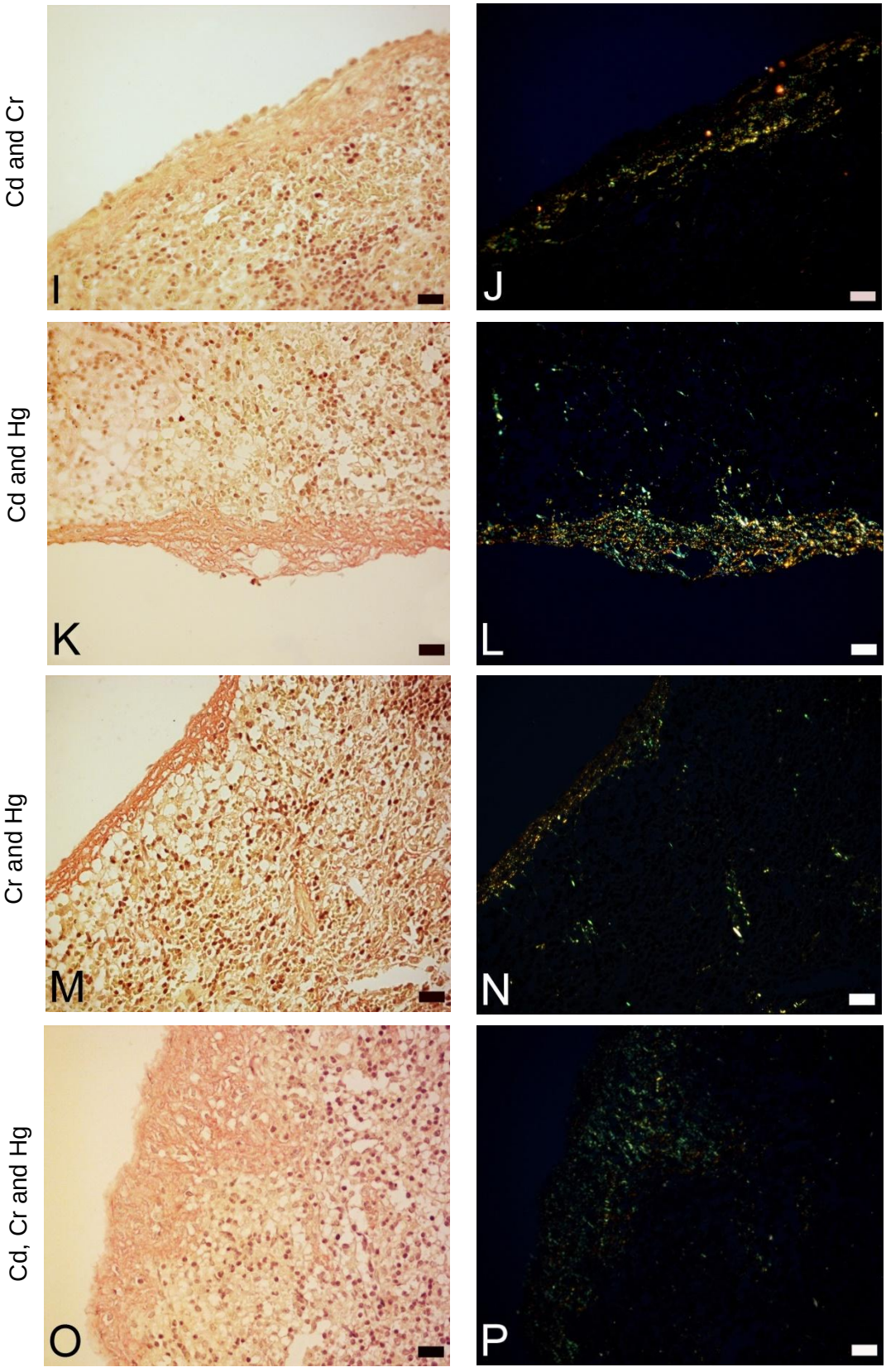

Fig. 4

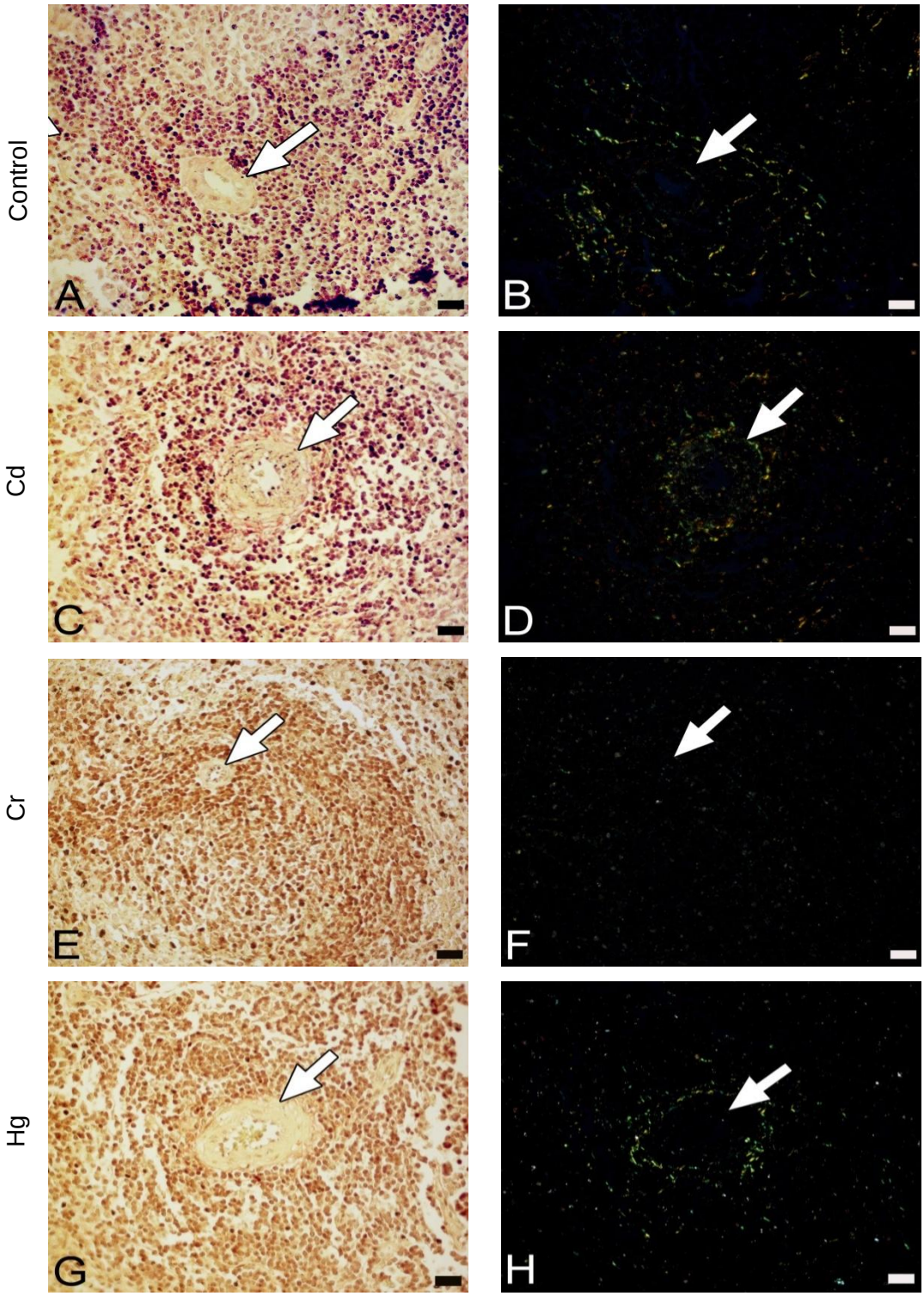

Fig. 5

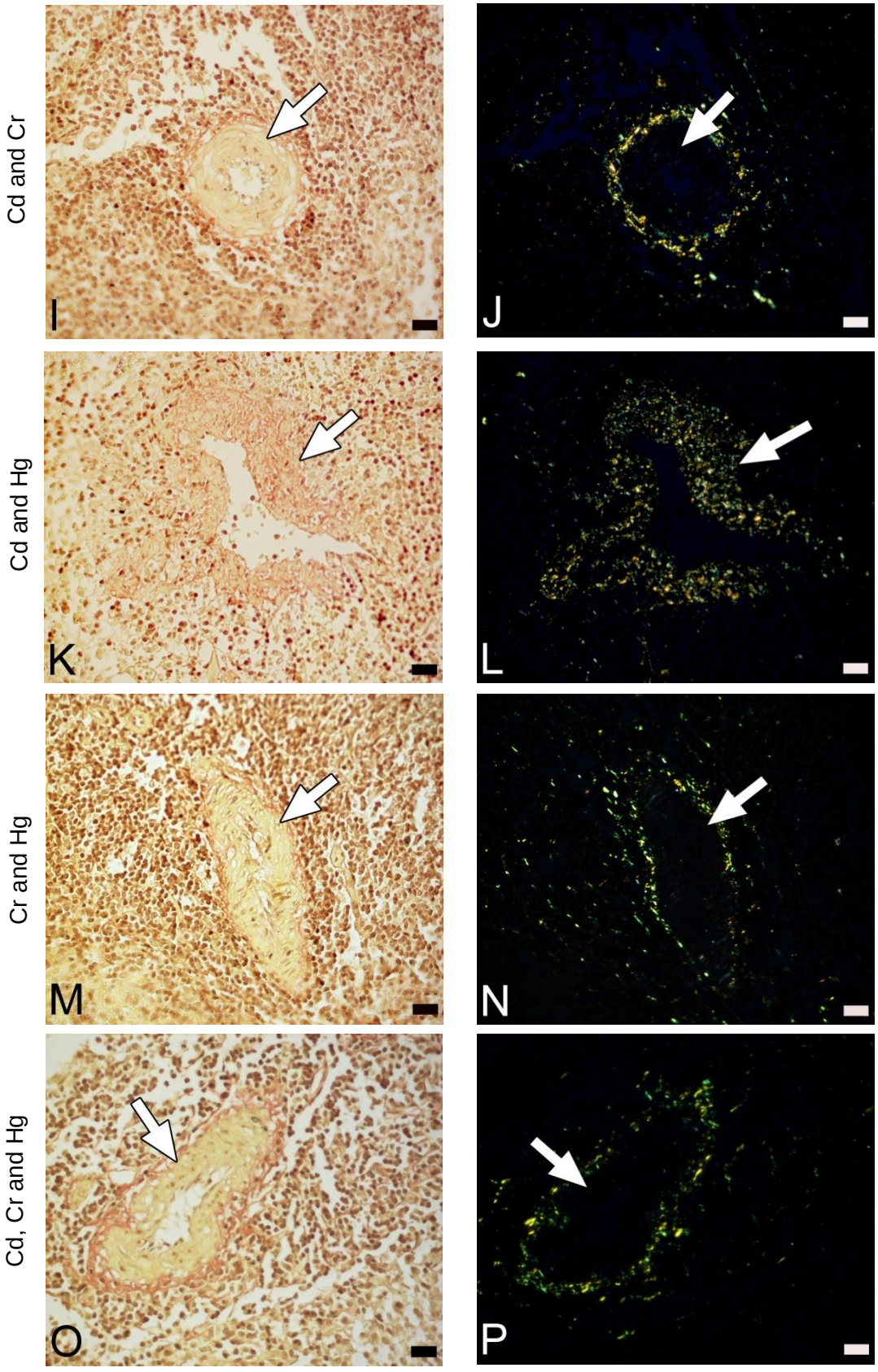

Fig. 6

FIGURE LEGENDS

Figure 1: Light micrographs of the spleen tissue from the control and single metal exposure (Cd, Cr and Hg) groups. Figures A and B (Control) indicate no cellular alterations in the red- and white pulp respectively. Figures C and D (Cd) show minimal necrosis in the red- and white pulp. Figures E and F (Cr) show moderate necrosis in the red- and white pulp. Figures G and H (Hg) show severe necrosis in the red- and white pulp (black arrow). **Key:** Black arrows: Necrosis; White arrows: Central arteries; RP: Red pulp; WP: White pulp (Scale bars: A-H: 50μm). H&E staining.

Figure 2: Light micrographs of spleen tissue from the double and triple combination groups. Figures I and J (Cd and Cr), Figures K and L (Cd and Hg), Figures M and N (Cr and Hg) and Figure O and P (Cd, Cr and Hg) shows severe necrosis in the cells of the red- and white pulp (black arrow). **Key:** Black arrows: Necrosis; White arrows: Central arteries; RP: Red pulp; WP: White pulp (Scale bars: I-P: 50μm). H&E staining.

Figure 3: Light micrographs of the capsule of the spleen from the control (A and B), Cd (C and D), Cr (E and F) and Hg (G and H) groups. Collagen was present in the capsule and trabeculae extending into the red pulp. Figures A, C, E and G are the bright field micrographs and B, D, F and H are the polarized micrographs (Scale bars: A–H: 20μm). PR staining.

Figure 4: Light micrographs of the capsule of the spleen from the Cd and Cr (A and B), Cd and Hg (C and D), Cr and Hg (E and F) and Cd, Cr and Hg (G and H) groups, with moderate to severe amounts of collagen present in the capsule and trabeculae extending into the red pulp.

Figures I, K, M and O are the bright field micrographs and J, L, N and P are the polarized micrographs (Scale bars: A–H: 20μm). PR staining.

Figure 5: Light micrographs of the spleen central arteries of the control (A and B), Cd (C and D), Cr (E and F) and Hg (G and H) groups. Minimal to moderate amounts of collagen deposition were found surrounding the central artery (arrows) of the control, Cd and Hg groups, with no collagen depositions found in the Cr group. Figures A, C, E and G are the bright field micrographs and B, D, F and H are the polarized micrographs. **Key:** Arrows: Central arteries (Scale bars: A–H: 20μm). PR staining.

Figure 6: Light micrographs of the spleen central arteries of the Cd and Cr (A and B), Cd and Hg (C and D), Cr and Hg (E and F) and Cd, Cr and Hg (G and H) groups. Moderate to severe amounts of collagen deposition were found surrounding the central artery (arrows). Figures A, C, E and G are the bright field micrographs and B, D, F and H are the polarized micrographs. **Key:** Arrows: Central arteries (Scale bars: A–H: 20μm). PR staining.