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New predictive tools for liver fibrosis among non-apparently contaminated heavy metal workers

Nabil Mohie Abdel-Hamid ¹, Mona Mohammed Abdel Hamid ², Ahmed Abd El-Naser Mohamed ^{3*}, Ahmed Hassan Abd El-Salam Mohamed ⁴

¹ Department of Biochemistry, Faculty of Pharmacy, Kafrelsheikh University, Egypt

² Department of Biochemistry, Faculty of Pharmacy (for Girls), Al-Azhar University, Egypt,

Orcid ID: 0000-0002-2810-7016.

^{3*} Department of Biochemistry, Faculty of Pharmacy, New Mansoura University, Egypt,

Orcid ID: 0000-0003-0666-234X.

⁴ Department of Forensic Medicine and Clinical Toxicology, Faculty of Medicine (for Boys), Al-Azhar University, Egypt.

* **Correspondence:** Ahmed Abd El-Naser Mohamed. Department of Biochemistry, Faculty of Pharmacy, New Mansoura University, Egypt.

E-mail: Ahmedabdelnaser112@gmail.com, Ahmed.abdelnaser@nmu.edu.eg

Orcid ID: 0000-0003-0666-234X.

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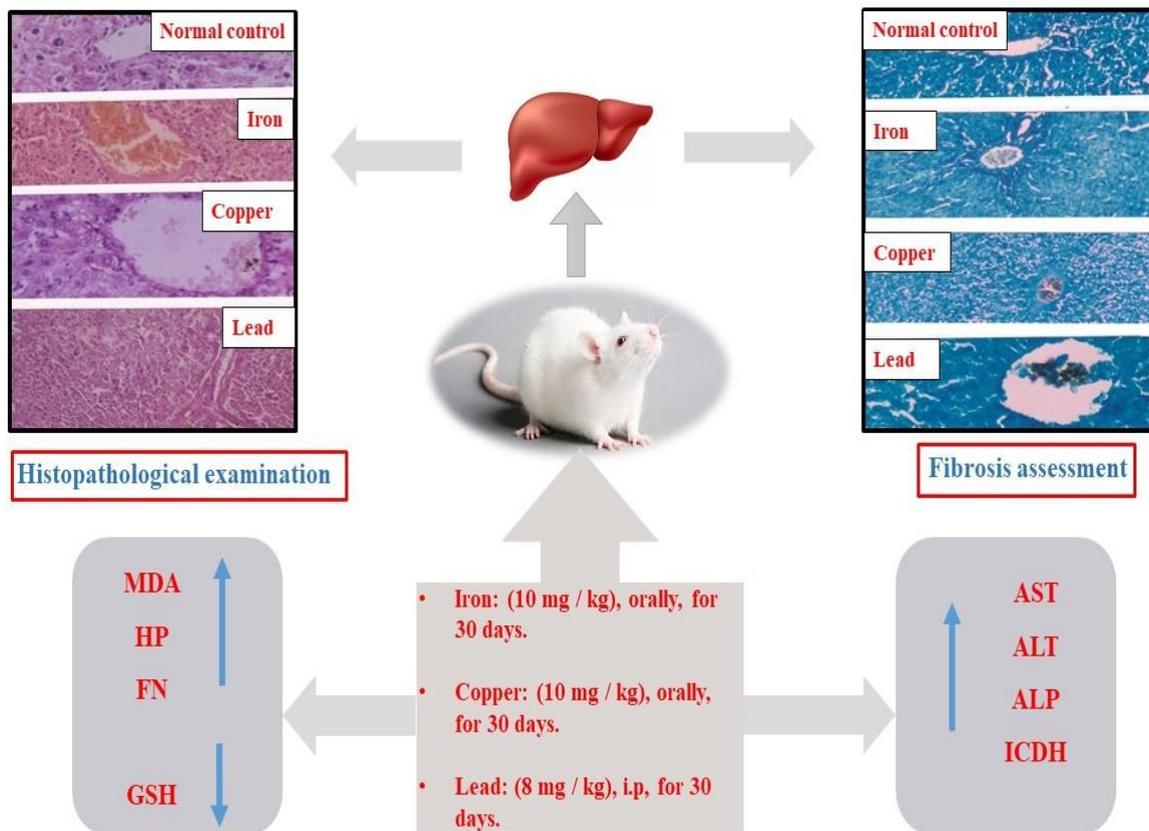
Abstract

Background and aim: Accumulation of heavy metals, such as iron, copper, and lead can be hepatotoxic. This study aimed to investigate the effect of exposure to iron, copper, and lead on the liver, along with an experimental study. **Methods:** Thirty-six male workers from 3 factories in Egypt (12/each), lead, copper, and iron groups against a control group. Thirty-two rats were classified into 4 groups (8/each), control, iron (given oral ferrous gluconate, 10 mg/kg body weight), copper (given oral copper sulfate, 10 mg/kg body weight), and lead (given intraperitoneal lead acetate, 8 mg/kg body weight) daily for 30 days. **Results:** Human iron, copper, and lead serum levels were non-significantly higher than control. Serum total glycosaminoglycans (TGAGs) and fibronectin (FN) were significantly raised in all workers with significant increases in alkaline phosphatase in iron and copper workers, and aspartate aminotransferase in ironworkers. Experimentally, liver hydroxy proline was significantly elevated, with disturbed tissue oxidative stress, serum liver indices with depressed hepatic tissue TGAG, and fibrosis among metal-loaded rats. **Conclusion:** The studied workers seem protected against metal intoxication, but non-significant serum increments could predict future hepatic fibrosis, manifested as correlative elevations in serum TGAGs and FN. Disrupted experimental fibro-genic parameters could explain our theory.

Keywords: Hepatotoxicity; Heavy metals; Fibrosis; Oxidative stress; Hepatotoxicity.

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Graphical Abstract:



Introduction

The liver is the main target organ for metalloids toxicity. Though numerous pathways could be implicated in heavy metal-induced organ injury; oxidative stress is a critical mechanism for cytotoxicity induced by metal pollutants (Ommati & Heidari, 2021). Heavy metals have a serious risk if they exceed permissible limits in the body. Many heavy metals such as lead, chromium, arsenic, mercury, nickel, and cadmium have cumulative hepatotoxicity (Renu et al., 2021).

The concentration and proportion of heavy metals vary depending on the environment and

geographical location, so different locations have varying levels of exposure to heavy metals (Fiati Kenston et al., 2018). Heavy metal contamination emerged as a result of the rise of industrialization and human impacts (Xiao-san, Shen, Yong-guan, & Xiang-dong, 2012).

Raised iron level is a common feature of all these fibrosis-promoting metals (Mehta, Farnaud, & Sharp, 2019). Although iron is crucial for normal physiology, excess iron is poisonous as it can accelerate the Fenton reaction that produces noxious reactive oxygen species (ROS) and severely harms cells and tissues (Mehta et al., 2019).

Chronic copper toxicity principally affects the liver because it is the first location of copper deposition after it arrives in the blood. Extra copper seems to display toxicity by the generation of free radicals, which result in lipid peroxidation, depletion of antioxidants, and polymerization of Cu-thionine, causing cellular necrosis and apoptosis (Poujois, Poupon, & Woimant, 2019).

Due to immunological modulation, oxidative, and inflammatory pathways, Pb exposure can cause different diseases such as liver disease. Additionally, Pb has the potential to disrupt the oxidant-antioxidant system's balance and cause inflammatory reactions in a variety of organs (Balali-Mood, Naseri, Tahergorabi, Khazdair, & Sadeghi, 2021).

Free radicals oxidize unsaturated fatty acids in biomembranes during lipid peroxidation. Malondialdehyde (MDA) is an indicator of oxidative stress and one of the end-products of lipid peroxidation (Mohamed et al., 2022). Excess in free radicals means overproduction of MDA and the reduced GSH levels in cirrhotic patients can increase cellular damage. GSH level is also reported to decrease owing to increased oxidative injury in hepatic cells (Aydin, Dirik, Demir, Tolunay, & Demir, 2021). The existence of hydroxyproline (HP) in the extracellular matrix (ECM) generated by activated hepatic stellate cells (HSCs) maintains the entity and function of liver cells. Its level in liver tissues provides a regulating factor that could indicate properly the degree and progression of liver fibrogenesis (Gabr, Alghadir, Sherif, & Ghfar, 2016). Nabil et al. reported a significant positive correlation between hydroxyproline and MDA, and a significant negative correlation between HP and GSH (Nabil, Ali, Shiha, & Zahran, 2021).

Glycosaminoglycans (GAGs) are implicated in a large number of bio-processes and perform an important role in growth and development, preserving homeostasis, and resisting disease (Shi, Sheng, & Chi, 2021). The GAGs concentration was

significantly increased in the blood samples of patients with cirrhosis, nonalcoholic fatty liver disease, and hepatocellular carcinoma (Guo et al., 2015). Elevated fibronectin (FN) expression has an essential role during liver fibrosis. Since FN seems to have a fundamental role in liver fibrogenesis, its expression may be reflected as a critical factor in mediating the long-term concerns of several chronic liver disorders (Liu et al., 2016). FN was observed as a potential diagnostic biomarker for HCC. The diagnostic performance of serum fibronectin exceeded that of the current biomarker, α -fetoprotein (AFP), for early-stage HCC after liver cirrhosis (Kim et al., 2020). As well, FN is a non-invasive indicator for the evaluation of liver fibrosis in chronic HCV patients (Ghafar et al., 2019).

In the current study, we aimed to investigate the possible hepatotoxicity and oxidative stress upon chronic exposure to heavy metals such as iron, copper, and lead among workers working for a long time in front of these metal ovens in selected Egyptian metal factories. Blood levels of these metals were estimated for these individuals along with monitoring FN, GAGs, and liver enzyme activities such as ALT, AST, ALP, and isocitrate dehydrogenase (ICDH). Parallel to this clinical investigation; experimental work was applied to mirror the effect of the same metals on rats after daily use for a month. This later experimental work enabled us to correlate our clinical findings to non-available tissue testing, as the workers looked healthy and liver biopsies were impossible to assume for them. So, in addition to histopathological tests, we evaluated tissue oxidative changes as MDA and GSH contents in rats with liver enzymes.

Material and methods

Acceptance of the research committee at the Faculty of Pharmacy, Kafrelsheikh University was attained for the study, respecting the animal and human research guidelines. The experimental procedures of the study were performed according to ARRIVE guidelines in agreement with the UK. Animals

(Scientific Procedure) Act, 1986 and associated guidelines. (Approval code: KFS-ph-01-22).

Experimental design

Part I: Human study

Human subjects: Forty-eight male healthy individuals of an average of 40 years of age were selected from different locations and divided into 4 groups (12 of each). The metal oven workers were working for 10 - 20 years. The studied individuals were classified as follows:

Control group: Healthy individuals from different locations were recruited as a normal control. Helwan, South of Cairo, Egypt.

Lead group: Lead-exposed workers, in lead melting unit, Helwan Factory for Diesel Engines,

Copper group: Copper-exposed workers, in copper melting unit, National Copper Company, Alexandria, Egypt.

Iron group: Iron-exposed workers, in an iron melting unit, Shobra El-Khema, North of Cairo, Egypt.

Informed consent was taken from all individuals after presenting a seminar about the objectives of the study to show to what extent they are protected against metal intoxication.

Blood Sampling

Fasting blood samples were collected in the morning, centrifuged and sera were kept frozen at -80°C right biochemical investigations.

Part II: Animal study

Chemicals

Ferrous gluconate (Merck, Germany), Copper sulfate (Merck, Germany), and Lead acetate (El-Nasr, Egypt), were separately dissolved in sterile distilled water. Both Fe and Cu salts were given orally (P.O) by gastric gavage while Pb salt was given intraperitoneally (IP). All used chemicals were of analytical grade.

Animals

The experiment was carried out on thirty-two male albino rats with an average weight of 190 ± 20 g.

Rats were obtained from the Animal Farm at the Faculty of Veterinary Medicine, Zagazig University, Egypt. Rats were kept in polyethylene cages under constant environmental conditions and observed daily throughout the study period. They were kept at a constant temperature of $25 \pm 2^{\circ}\text{C}$ with enough ventilation at a 12-hour cycle of light and dark, and unrestricted access to drink and food.

Experimental design for animals

After 7 days-accommodation, thirty-two rats were used and classified into 4 groups (8 rats each) as follows:

Control group: rats received a daily volume of normal saline orally.

Iron group: The rats received a single P.O. daily dose of ferrous gluconate daily (10 mg/kg body weight) (Bisse, Renner, Sussmann, Schölmerich, & Wieland, 1996).

Copper group: The rats received a single P.O. daily dose of copper sulfate (10 mg/kg body weight) (Kumar, Sathua, & Flora, 2019).

Lead group: The rats received a single IP daily dose of lead acetate (8 mg/kg body weight) (Thoreux-Manlay, Le Goascogne, Segretain, Jégou, & Pinon-Lataillade, 1995). The study period was 30 days.

Collection of the biological samples

Clean capillary tubes were utilized to collect blood samples by retro-orbital puncture of each rat under light ether anesthesia by the end of the experiment. Upon clotting and centrifugation at 3000 rpm for 15 minutes, sera were instantly frozen at -80°C right biochemical investigations. Afterward, rats were sacrificed via cervical decapitation, and their livers were removed, washed with saline, dried with filter papers, weighed, and split into two parts: the first was immediately immersed in 10% buffered formal saline for histological evaluations, and the second was directly homogenized in buffered saline (10% w/v), filtered and the supernatant was stored at -80°C right biochemical analyses.

Histopathological examination of the liver tissue

Liver specimens were placed in ascending degrees of ethanol for dehydration after being submerged in 10% buffered formalin. They were encased in paraffin wax and sliced into 5 μ thick samples. For tissue structure analysis under a light microscope, sections were stained with Mayer's hematoxylin and eosin (H&E) stains. Another number of slides was stained with Alcian blue solution in acetic acid. Histological alterations were photographed using a computer system with a digital camera (Nikon digital camera, Japan).

Biochemical analysis for both human and experimental samples

Serum enzyme activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were spectrophotometrically measured based on Reitman and Frankel method (Reitmann, 1957). Serum alkaline phosphatase (ALP) and isocitrate dehydrogenase (ICDH) activities were measured by kinetic assay according to Jordan (Jordan, 1966) and Yamada, *et al.*, (Yamada *et al.*, 2014) respectively. Serum total glycosaminoglycans were measured by the colorimetric method according to Schloss, (Schloss, 1951). Plasma fibronectin (FN) level was measured by radial immunodiffusion (RID) procedure according to Mancini *et al.*, (Mancini, Carbonara, & Heremans, 1965) and Fahey and McKelvey, (Fahey & McKelvey, 1965). Serum concentrations of lead and copper were determined by atomic absorption/flame spectrophotometer (Shimadzu, Model AA- 640 - 13, Japan) following the procedures of De Silva, (DeSilva, 1981) and Parker *et al.*, (Parker, Humoller, & Mahler, 1967) respectively. The serum level of iron was measured

spectrophotometrically according to the Dreux procedure (Dreux, 1977). Liver homogenates were used for the determination of hydroxyproline (Woessner Jr., 1961), MDA (Ohkawa, Ohishi, & Yagi, 1979), GSH (Beutler, 1963), and malondialdehyde (MDA)(Li & Chow, 1994).

Results

Animal study:

After 30 days of administrations, significant variations of serum enzyme levels were observed in the different studied groups (Table. 1). Significant increase in serum levels of AST in both copper and lead groups in comparison to the control group ($P < 0.01^{**}$ and $P < 0.001^{***}$, respectively). Also, ALT and ALP showed significant increases in all serum levels of the studied biomarkers in the different sets in comparison to the control group except in the lead group which ALP level was reduced with significant differences ($P < 0.05^{*}$ and $P < 0.001^{***}$). Regarding GAGs, a significant increase in the serum levels in iron and lead groups in comparison to the control group ($P < 0.001^{***}$).

Moreover, as shown in the table. 1, GSH content in the rat's liver homogenates after 30 days' administration revealed a significant decrease in comparison to the control group ($P < 0.001^{***}$), while MDA and HP contents showed a significant increase in all the studied groups in comparison to the control group ($P < 0.01^{**}$ and $P < 0.001^{***}$).

As shown in **Table 2**, a positive significant correlation was detected between liver HP and MDA in 30-day lead-treated rats. Furthermore, the liver content of GSH and MDA of the same group was negatively correlated.

Table 1: Serum and tissue parameters of the animal study:

Parameters	Control	Iron (10mg/kg)	Copper (10mg/kg)	Lead (8mg/kg)
Serum parameters				
AST (U/l)	28.3 ± 1.0	30.6 ± 1.4	34.4 ± 1.1**	37 ± 1.4***
ALT (U/l)	19.8 ± 0.75	25.4 ± 0.6***	26.6 ± 0.7***	22.4 ± 0.82*
ALP (U/l)	114 ± 3.4	134 ± 2.1***	112.2 ± 2.7	104 ± 2.1*
GAGs (µg/ ml)	86.8 ± 1.9	140.5 ± 4.8***	92.6 ± 6.0	151 ± 6.7***
Tissue parameters				
GSH (µg/ g tissue)	4240 ± 33	2944 ± 125***	2096 ± 19***	3698 ± 37***
MDA (ng/g tissue)	137 ± 4.4	173.8 ± 10.9**	178.9 ± 40***	223.8 ± 8.4***
HP (µg/ g tissue)	8.4 ± 0.31	31.4 ± 1.3***	38.14 ± 0.93***	38.7 ± 0.62***

Data was expressed as mean ± SEM and are considered a significant than the control group at $P < 0.05^*$, $P < 0.01^{**}$, and $P < 0.001^{***}$.

Table 2: Correlations between the studied biomarkers in the different studied rats groups:

Parameters	r	P value
HP and MDA in 30 days lead treated rats	+ 0.40	< 0.05
GSH and MDA in 30 days lead treated rats	- 0.23	N.S

Administration of ferrous gluconate and copper sulfate induced certain congestion of the central vein (C.V) in association with different degrees of fatty changes (**Fig. 1: 1,2**). Lead acetate induced in addition, marked tissue fibrosis (**Fig. 1, 3**). An increase in chondroitin sulfate deposition around the C.V. that decreases towards the periphery was shown by treatment with these metals (**Fig. 2**).

Histopathological Examination

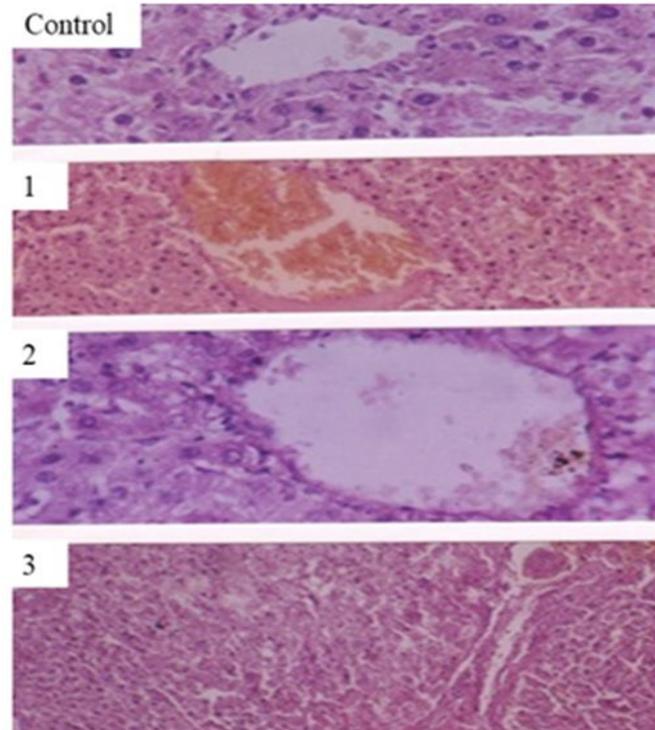


Figure 1: Shows the effect of iron, copper and lead on rat liver tissue after one month of administration. (1) normal control. (2) iron. (3) copper. (4) lead. (H&E stain, X 300).

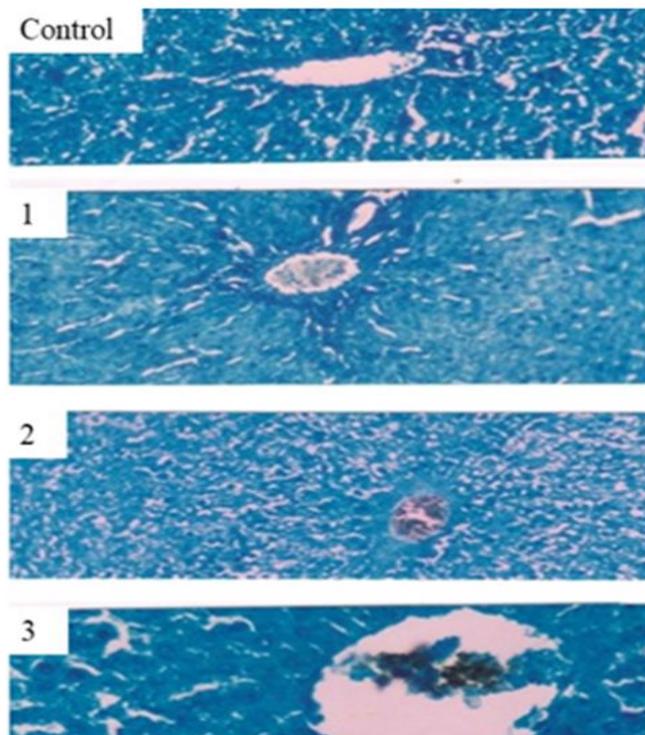


Figure 2: Shows the effect of iron, copper and lead on GAGs distribution around the C.V in the liver of rats after one month of administration. (1) normal control. (2) iron. (3) copper. (4) lead. (Alcian blue stain) X 300).

Human study:**A. Serum level of metals:**

As shown in the **table. 3**, Serum levels of metals (lead, copper, and iron) in oven workers showed slight increases in comparison to the control group but with no significant difference.

B. Serum biochemical parameters in metal oven workers compared to normal control:

As shown in the **table. 4**, Alkaline phosphatase (ALP) activity was significantly increased in iron and copper oven workers in comparison to the normal control group ($P < 0.01$ and $P < 0.001$, respectively). No significant change was seen in the

activities of AST, ALT, ALP, and ICDH in lead oven workers in comparison to the normal control group. The ironworkers' group showed a significant increase in AST activity in comparison to the normal control group at $p < 0.01$.

Moreover, a significant increase in serum GAGs levels was observed in all workers exposed to metal vapors in comparison to the normal control group at $P < 0.001$. Similarly, a significant increase in fibronectin levels was observed in iron, copper, and lead workers' groups in comparison to the normal control group at $P < 0.001$, $P < 0.01$ and $P < 0.001$, respectively.

Table 3: Serum iron, copper, and lead levels in metal oven workers compared to control. Values are expressed in terms of mean \pm S.E.M.

Iron (mg /l)		Copper (mg/l)		Lead (mg/l)	
Control	Workers	Control	Workers	Control	Workers
1.6\pm 0.1	1.8\pm 0.1	2.15\pm0.04	2.17\pm0.004	0.77\pm0.006	0.78\pm0.006

Table 4: Serum parameters of the human study:

Parameters	Control	Iron workers	Copper workers	Lead workers
AST (U/l)	9.2 \pm 0.4	11.6 \pm 0.5**	10.4 \pm 0.5	8.7 \pm 0.5
ALT (U/l)	8.0 \pm 0.4	8.9 \pm 0.6	9.3 \pm 0.7	9.0 \pm 0.4
ALP (U/l)	26.4 \pm 0.3	29.0 \pm 0.7**	29.9 \pm 0.43***	26.4 \pm 0.8
ICDH (U/l)	9.8 \pm 0.4	10.0 \pm 0.4	9.9 \pm 0.3	9.7 \pm 0.4
GAGs (μ g/ ml)	281.7 \pm 2.0	558.3 \pm 4.0***	473.3 \pm 3.0***	585.6 \pm 1.89***
Fibronectin (mg/l)	240 \pm 4.0	344.2 \pm 1.0***	321.3 \pm 4.0**	259.6 \pm 5.0***

Values are expressed as mean \pm SEM and are considered significant in comparison to the control group at $P < 0.05^*$, $P < 0.01^{**}$, and $P < 0.001^{***}$.

Table 5: Correlations between the studied biomarkers in the different studied human groups:

Parameters	r	P value
GAGs and fibronectin in iron oven workers	+ 0.21	< 0.05

GAGs level was positively correlated with fibronectin in iron oven workers.

Discussion

Numerous heavy metals can enter the human body at the same time via the air, water, or food. Heavy metals are not easily metabolized or excreted. Inside the body; they slowly accumulate causing their concentration to rise (Alves et al., 2017).

Hepatotoxicity was assessed by measuring hepatic oxidative stress and the levels of hepatic enzymes such as AST, ALP, and ICDH.

In our study, the iron group showed a negligible elevation of serum Fe level among iron workers, along with a significant increase in serum AST and ALP activities and TGAGs' levels in comparison to the normal control group. Although the increments in AST and ALP activities fall within clinically-acceptable levels; these results showed a disagreement with a previous study reported by **Heris et al.**, who stated that liver enzyme activities were significantly elevated in iron-overloaded humans (Heris et al., 2021). Both ALT and ICDH showed a non-significant up-regulation in their activities, while FN was significantly up-regulated. The elevated FN levels are possibly non-prognostic to these patients and can predict liver fibrosis despite the negligible elevations in serum Fe levels (Younesi & Parsian, 2019).

Although Cu is a necessary metal for humans and animals; excessive exposure can be harmful to human health. The World Health Organization

(WHO) acknowledged the difficulty in determining copper intake limits (Chemicals, 2002).

In the present work, the Cu group showed a marked increase in serum ALP activity, GAGs, and FN levels along with non-significant serum Cu elevation in comparison to the normal control group. Tian et al. found that Cu overload has a significant role in the induction of hepatotoxicity marked by increased liver enzyme activities (Tian et al., 2019). The elevations of both FN (Younesi & Parsian, 2019) and TGAGs (Gressner, Köster-Eiserfunke, Van de Leur, & Greiling, 1980) are suggestive of emerging liver fibrogenesis despite the existence of non-significant metal increments.

In our study, the Pb group displayed non-significant increases in the studied serum enzyme activities with significant elevations in FN and TGAG levels. Once again, the increased serum FN (Younesi & Parsian, 2019) and TGAGs (Gressner et al., 1980) seem to be predictive of future liver fibrogenesis despite the existence of non-significant metal increments. This is also clear from the apparently significant positive correlation between TGAGs and fibronectin in iron oven workers.

Liver fibrosis is caused by persistent liver damage along with the accumulation of extracellular matrix (ECM) proteins, which is a common feature of most chronic liver disorders. Fibrosis always emerges after an accumulation of ECM proteins disrupting the hepatic architecture by establishing a

fibrous scar (Aydın & Akçalı, 2018). Heavy metal hepatotoxicity is believed to be responsible for fibrosis occurrence through the increased level of HP in hepatic tissues. We executed experimental work on heavy metal-intoxicated rat groups to figure out a better idea about tissue derangement responsible for tissue fibrosis. Histopathological examination of hepatic tissue of treated rats displayed congestion of the central vein (C.V) in association with different degrees of fatty changes.

In the current study, all studied groups showed marked elevation in hepatic HP content in comparison to the normal control group. These results were in agreement with Zhou et al, who revealed that iron was accumulated in the liver showing highly related liver fibrosis (Martínez-Peinado et al., 2018; Zhou, Tanabe, Fuchs, & Caravan, 2020). Moreover, it has been reported that elevated Pb contents were observed in hepatic cirrhosis (Reja, Makar, Visaria, Karanfiliyan, & Rustgi, 2020).

Oxidative stress has been linked to the pathogenesis of numerous disease circumstances and toxicities in animals. Upon xenobiotic exposure, cells produce reactive oxygen species and free radicals over their inherent capacity to neutralize them, resulting in oxidative stress, and protein, lipid, and DNA damage (Liguori et al., 2018). Interestingly, all groups showed a significant increase in hepatic MDA content and a decrease in GSH content in comparison to the normal control group.

Also, the use of heavy metals depleted TGAGs in the hepatic tissue, especially around the C.V. Disrupted liver tissue HP and TGAGs was previously reported as a sign of possible persistent tissue changes that may lead eventually not only to fibrosis but can lead to hepatocellular carcinoma

(Abdel-Hamid, 2009). These results suggest the role of iron, copper, and lead accumulation in hepatotoxicity induction (Mitra et al., 2022).

Conclusion

Chronic exposure to heavy metals such as iron, copper, and lead, although may not lead to significantly elevated serum levels, some hidden pathophysiological aberrations such as disturbed serum FN and TGAGs, along with disrupted tissue distribution of GAGs and antioxidant mechanisms may eventually correlate to liver tissue fibrosis.

Abbreviations: ALP: alkaline phosphatase; ALT: alanine aminotransferase; AST: aspartate aminotransferase; Cu: copper; FN: fibronectin; TGAGs: total glycosaminoglycan; GSH: reduced glutathione; HP: hydroxyl proline; ICDH: isocitrate dehydrogenase; MDA: malondialdehyde; ROS: reactive oxygen species.

Authors Contributions:

NMA: Conceptualized the work idea. Supervised the practical work. Revised the whole MS and submission. **ME and AHM:** Executed part of the experiments and biostatistics. **AA M:** Executed part of the experiments, wrote the results and drafted the MS. The authors declare that all data were generated in-house and that no paper mill was used.

Conflict of interest

All authors declare that there is no conflict of interest.

Fund

No fund was obtained.

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