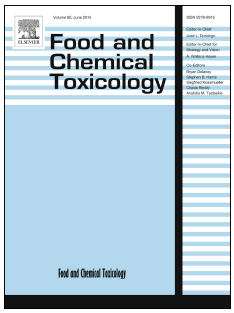

Transcriptional and biochemical changes in mouse liver following exposure to a metal/drug cocktail. Attenuating effect of a selenium-enriched diet

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PII: S0278-6915(24)00411-3

DOI: https://doi.org/10.1016/j.fct.2024.114845

Reference: FCT 114845

To appear in: *Food and Chemical Toxicology*

Received Date: 8 April 2024

Revised Date: 13 June 2024

Accepted Date: 27 June 2024

Please cite this article as: Huertas-Abril, P.V., Prieto-Álamo, M.-J., Jurado, J., Pérez, J., Molina-Hernández, V., García-Barrera, T., Abril, N., Transcriptional and biochemical changes in mouse liver following exposure to a metal/drug cocktail. Attenuating effect of a selenium-enriched diet, *Food and Chemical Toxicology*, https://doi.org/10.1016/j.fct.2024.114845.

Graphical Abstract

Huertas-Abril et al., Pollutants interact…

Transcriptional and biochemical changes in mouse liver following exposure to a metal/drug cocktail. Attenuating effect of a selenium-enriched diet

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Abstract

Real-life pollution usually involves simultaneous co-exposure to different chemicals. Metals and drugs are frequently and abundantly released into the environment, where they interact and bioaccumulate. Few studies analyze potential interactions between metals and pharmaceuticals in these mixtures, although their joint effects cannot be inferred from their individual properties. We have previously demonstrated that the mixture (PC) of the metals Cd and Hg, the metalloid As and the pharmaceuticals diclofenac (DCF) and flumequine (FLQ) impairs hepatic proteostasis. To gain a deeper vision of how PC affects mouse liver homeostasis, we evaluated here the effects of PC exposure upon some biochemical and morphometric parameters, and on the transcriptional profiles of selected group of genes. We found that exposure to PC caused oxidative damage that exceeded the antioxidant capacity of cells. The excessive oxidative stress response resulted in an overabundance of reducing equivalents, which hindered the metabolism and transport of metabolites, including cholesterol and bile acids, between organs. These processes have been linked to metabolic and inflammatory disorders, cancer, and neurodegenerative diseases. Therefore, our findings suggest that unintended exposure to mixtures of environmental pollutants may underlie the etiology of many human diseases. Fortunately, we also found that a diet enriched with selenium mitigated the harmful effects of this combination of toxicants.

Keywords: pollutant cocktail; metals/drug mixture; hepatotoxicity; absolute qRT-PCR; oxidative/reductive stress; Se-enriched diet.

1. Introduction

The continuous exposition of the human population to chemicals generates serious concern worldwide derived from the well documented relationship between exposure and disease (González et al. 2019). The chemical risk derives not only from the toxicity of these compounds but also from their persistence and capacity for deposition in body tissues, which leads to their accumulation along the trophic chain and the biomagnification of their effects (Peters et al. 2022). Heavy metals are part of this group of pollutants whose presence in the ecosystem is one of the most serious and widespread environmental problems today (Rehman et al. 2018). These elements are generally non-biodegradable, highly resistant to conventional disposal treatments, and toxic due to their ability to substitute essential elements in the active site of some enzymes and for generating oxidative stress in cells (Carneiro et al. 2018). In recent years, there has also been increasing concern about the presence of pharmaceuticals in the natural environment. The environmental concentration of these emerging pollutants is usually low enough to be considered non-toxic. However, a large body of evidence suggests that they may cause endocrine, epigenetic and developmental changes in aquatic organisms and in humans under conditions of prolonged exposure [e.g., (Wilkinson et al. 2016)].

Epidemiological and experimental studies indicate that metals and pharmaceuticals co-occur within the environment and interact in a manner that may both increase or decrease their impact on human health [e.g., (Fiati Kenston et al. 2018, Lin et al. 2016, Matejczyk et al. 2020)]. However, the studies analyzing the potential synergistic/antagonistic interactions between these two groups of chemicals are scarce, despite co-exposure is a frequent event, as they are released into the environment from multiple anthropogenic activities and bioaccumulated by organisms (Chormare &Kumar 2022, Nagpal et al. 2018, Nilsen et al.

2019, Zenker et al. 2014). We have previously evaluated the toxicity of a mixture of metals/metalloids and pharmaceuticals that commonly contaminate food and water (Fekadu et al. 2019, González-Gaya et al. 2022, Thompson &Darwish 2019). All the components (arsenic, cadmium, mercury, diclofenac, flumequine) of our pollutant cocktail (PC) have been selected because of their abundance and dangerous effects on ecosystems and their inhabitants, including humans. Arsenic (As) is a ubiquitous, naturally occurring metalloid element that may enter the body through dermal contact, ingestion, or inhalation. Even at low concentrations, As can cause negative health consequences, from inflammation to cancer [(Shiek et al. 2023) and references herein]. Cadmium (Cd), used in various industrial activities, has an extremely long biological half-life (approximately 20-30 years in humans), a low excretion rate from the body, and is predominantly stored in soft tissues (mainly liver and kidney) (Rani et al. 2014). Diet is the second most important cause of exposure to Cd after cigarette smoking, and dietary exposure to cadmium has been linked to liver and kidney damage, bone demineralization, and thyroid and metabolic disease [reviewed in (Schaefer et al. 2020)]. Mercury (Hg) is also a ubiquitous and harmful metal that occurs naturally in the Earth's crust and whose environmental levels have been greatly increased by human activities in recent centuries. This increase is of concern to public health due to the known toxic effects of Hg exposure on human health (Munthe et al. 2019).

Diclofenac (DCF) is a widely utilized pharmaceutical for its analgesic, anti-inflammatory, and antipyretic properties (Gan 2010). Due to its high usage and low removal rate in water treatment processes, DCF is one of the most ubiquitous pharmaceuticals detected in aquatic and soil environment (He et al. 2017), reaching average environmental concentrations of almost mg/L level (Li et al. 2023). Diclofenac has garnered increased attention due to its harmful potential (Li et al. 2023), as DCF exposure can result in a number of serious side effects, including renal damage, liver toxicity, gastrointestinal damage, and

increased cardiovascular risks (Elbaz et al. 2022). Flumequine (FLQ) is a synthetic fluoroquinolone antibiotic mainly active against Gram-negative bacteria. This antibiotic is used in intensive farming to control animal diseases, resulting in massive use and a considerable environmental load of the antibiotic and/or its metabolites (Ungemach et al. 2006). Increasing data exists on FLQ adverse effects in humans, with serious and disabling consequences (e.g. renal toxicity, tendinopathies, development of bacterial resistance) (Tennyson &Averch 2017).

The results of our previous studies showed that PC significantly alters the taxonomic structure of the gut microbiome and affected the mouse plasma (Arias-Borrego et al. 2022) and brain (Parra-Martínez et al. 2022) metabolomes. Moreover, by using a thorough proteomic approach, we reported alterations in the mouse hepatic proteostasis indicating a sustained expression of the antioxidative response through NRF2 leading to reductive stress (RS) in the liver. In this scenario of RS, the synthesis of antioxidant enzymes was impaired, inflammation worsened, and cholesterol biosynthesis increased (Huertas-Abril et al. 2023). The present work aimed to investigate whether these alterations caused by PC exposure at the molecular level translate into histopathological liver damage and systemic changes, and to explore other possible consequences of RS.

The mechanism of toxicity of each of the five PC chemicals involves the generation of redox stress leading to oxidation of DNA, proteins and lipids, disruption of cellular membranes and impairment of mitochondrial function (Hazelhoff &Torres 2018, Jagadeesan &Pillai 2007, Kenmochi et al. 2007, Matovic et al. 2011, Mezynska &Brzoska 2019, Thai et al. 2023). Therefore, several studies demonstrated the efficacy of antioxidants like selenium (Se) in counteracting individual PC component toxicity. In example, Se provides a protective effect against Hg toxicity by covalently bonding with this element (Heath et al. 2010). Similarly, Se antagonizes the toxicity of As and Cd through sequestration of these

elements into biologically inert complexes and/or through the action of Se-dependent antioxidant enzymes (Zwolak 2020). Some studies also report that Se mitigates DCF-induced oxidative stress, inflammation, and hematological abnormalities in the liver and kidney of treated rats (Owumi &Dim 2019).

Reductive stress also led to net ROS production and oxidative cytotoxicity (Korge et al. 2015). Therefore, not surprisingly, we found that Se also attenuated the effects of the pollutants, reducing its negative effects on the plasma/brain metabolome and intestinal dysbiosis (Arias-Borrego et al. 2022, Parra-Martínez et al. 2022) and restoring, at least partially, the hepatic proteostasis in mice. The incorporation in this work of a mouse group fed with a diet supplemented with selenium will strengthen the suitability of using this essential element as a nutraceutical intervention able to prevent or ameliorate the consequences of human exposure to these pollutant mixtures, something that is currently difficult to avoid due to the high use of metals and pharmaceuticals and the inefficiency of water purification plants to remove them.

2. Material and methods

2.1. Bioethics, experimental design, and sample collection

This work was approved by the Animal Experimentation Ethical Committee of the University of Córdoba (UCO), and by the General Direction of Agricultural and Livestock Production (Regional Government of Andalusia, Ref. 02-01-2019-001) and complied with the ARRIVE guidelines, the European Community guidelines EU Directive 2010/63/EU for animal experiments and the Spanish Government Royal Decree 1386/2018. Animal handling was conducted by qualified staff in the Experimental Animal Facility of the UCO (SAEX).

Forty-eight male BALB/c mice (Charles River Laboratories), aged 8 weeks with an average body weight of 23-25 g, were randomly assigned to two groups. Mice in the control group (C, n=12) had unrestricted access to water and food (standard rodent chow with sodium selenite, Se, at 0.21 mg/kg) for the duration of the experiment (3 weeks). Mice in the pollutant cocktail (PC) groups (PC, n=20 and PC+Se, n=16) simultaneously received a mixture of metals (As, Cd, and Hg) in the drinking water and two drugs (flumequine, FLQ; diclofenac, DCF) in the chow during the last two weeks. The diet of PC+Se mice was additionally supplemented with Se (final concentration of 0.65 mg/kg) during exposure to PC (weeks 2 and 3) (More details in the Suppl. Material & Methods section). Suppl. Table 1 lists the compounds in PC and the doses used.

2.2. Histopathological analyses

Liver samples were immersed in 10% buffered formalin for fixation and a standard histological processing procedure with hematoxylin-eosin (H-E) was followed for histopathological evaluation (More details in the Suppl. Material & Methods section).

2.3. Biochemical determinations in plasma

Plasma was used for quantification of aspartate transaminase (AST), alanine transaminase (ALT), total bile acids (BA), total and free cholesterol (CHO) and triglycerides (TAG), glucose (Glc), lactate (Lac), total protein (Prot), albumin (Alb), urea (UREA/BUN-UV) and creatinine (Creat). In all cases, commercial kits from BioSystems were used and the determinations were carried out on an Atom A-15 analyzer (BioSystems).

2.4. Determination of hepatic transcriptional profiles by real-time qRT-PCR.

2.4.1. RNA isolation from liver samples and cDNA synthesis

Total RNA was extracted with the commercial kit AllPrep® DNA/RNA/Protein (QIAGEN), following the manufacturer's instructions. Only RNAs with 260nm/280nm absorbance ratios of ~2.0 and RIN values greater than 8.5 were used. Synthesis of cDNA was carried out from 2 µg of total RNA isolated from each sample, using the iScript™ cDNA Synthesis Kit (BioRad), following the manufacturer's instructions, in a GeneAmp PCR System 9700 thermal cycler (PE Applied Biosystems).

2.4.2. Primers used to quantify *M. musculus* liver transcripts.

The primers used here are listed in Suppl. Table 2 and their amplification efficiencies were close to 100%.

2.4.3. Real-time qPCR amplification conditions

PCR reactions were carried out in triplicate using 50 ng of cDNA per reaction in a final volume of 20 µL and the SsoAdvanced Universal SYBR Green Supermix (BioRad) under the conditions indicated by the manufacturer.

In this work we performed absolute qRT-PCR for the quantification of the changes caused by the treatments on the transcript amounts of the target genes. The absolute quantification relates the PCR signal (C_T value in real-time PCR) to input copy number using a calibration curve (Prieto-Alamo et al. 2003). The calibration curve used in this study (C_T = - 3.32 x log N+ 39.69) was linear over 7 orders of magnitude and demonstrated a 100% PCR efficiency (explained in more detail in the Suppl. Material & Methods section).

2.5. Enzyme activity assays.

Proteins were quantified by the Bradford method (Bradford 1976) using the Protein Assay Dye Reagent Concentrate Reagent (BioRad), following the manufacturer´s protocol. Bovine serum albumin (BSA) was used to generate a standard curve to convert absorbance values into protein concentration.

The assay of superoxide dismutase (SOD) (McCord &Fridovich 1969), catalase (CAT) (Beers &Sizer 1952), glutathione peroxidase (GPX) (Flohé &Gümzler 1984) and glucose-6-phosphate dehydrogenase (G6PDH) (Löhr &Waller 1974), followed methods previously described with some modifications (explained in more detail in the Suppl. Material & Methods section).

2.6. Quantification of lipid peroxidation in the mice liver

Lipid peroxidation as malondialdehyde (MDA) was quantified by using the Cayman's TBARS (thiobarbituric acid-TBA-Reactive Substance) Assay Kit, following the manufacturer´s indications. Fluorescence was read at an excitation wavelength of 530 nm and an emission wavelength of 550 nm. A standard curve was generated with an MDA Standard solution contained in the kit. Total protein was estimated by the Bio-Rad Protein Assay method. The TBARS/MDA concentration was expressed in pmol/µg protein.

2.7. Quantification of NADPH, cholesterol, and bile acids in the mice liver

We used commercially available kits to fluorometrically measure the concentration of NADP/NADPH (Abcam, ab176724 NADP/NADPH Assay Kit) and total bile acids (Cell Biolabs, STA-631 Total Bile Acids Assay Kit) and colorimetrically determine the concentration of total and free cholesterol (Cell Biolabs, STA-384 Total Cholesterol Assay Kit) in liver tissue. In all cases we used a 96-well plate reader (Varioskan LUX Plate Reader, Thermo) and followed the manufacturer indications.

2.8. Statistical analysis

The statistical significance of the differences between the experimental samples was determined by a Dunnett's test with Bonferroni correction to compare the data of the different experimental groups with those of the control and a bilateral *t*-Student test to compare experimental groups with each other. The Dunnet test include the Bartlett statistic test for analyzing the homogeneity of variances and the Kolmogorov and Smirnov test for assessing normality. Both tools were included in the InStat software (*v*. 2.05/00, GraphPad) .

3. Results and Discussion

Assessing the toxicity of contaminants mixtures is essential since exposure to pollution experienced in "real life" includes combinations of several chemicals. In this study, we have evaluated the effects of a mixture (PC) of metals and pharmaceuticals on various biochemical parameters and the transcriptional abundance of a selected group of mouse liver genes. The components of the PC were chosen because of their abundance in water and food and the risk they pose to human health [e.g., (Carneiro et al. 2018, Elbaz et al. 2022, Gan 2010, Hazelhoff &Torres 2018, He et al. 2017, Hu et al. 2021, Li et al. 2023, Munthe et al. 2019, Rani et al. 2014, Schaefer et al. 2020, Shiek et al. 2023, Tennyson &Averch 2017, Ungemach et al. 2006, Wilkinson et al. 2016)]. In previous works we found that this particular mixture produced dysbiosis of the mice gut microbiota, alterations in the plasma and brain metabolome, and impairment of the hepatic proteostasis (Arias-Borrego et al. 2022, Huertas-Abril et al. 2023, Parra-Martínez et al. 2022). These previous results indicated that continued exposure to PC induced in the liver the so-called integrated stress response (ISR), an intracellular signaling network which results to a transient inhibition of global protein synthesis and the transcriptional reprogramming (Costa-Mattioli &Walter 2020). We also found that the mixture promoted the sustained NRF2 mediated expression leading to reductive stress (RS) by an excess of reducing equivalents. Much less studied than oxidative stress, but no less important, RS can affect cellular redox homeostasis, alter biomolecules (e.g., reduction of disulfide bonds in proteins and loss of their structure and/or function) and processes (e.g., membrane structure and function), and ultimately cause certain pathological conditions (Chatgilialoglu &Ferreri 2021). To gain a deeper vision of how IRS and RS affect mouse homeostasis, we considered it interesting to quantify the changes that continued exposure to PC induces in the expression, at the transcription level, of a selected group of genes involved in antioxidant defense and lipid metabolism, with particular interest

in the biosynthesis and transport of cholesterol and its derivatives bile acids, and to measure other parameters (liver histology, plasma biochemistry) that could reflect the consequences of PC exposure on liver function. In addition, we studied the ability of a selenium-enriched diet to attenuate the detrimental effect of this mixture of environmental pollutants.

3.1. Animals and treatments

Mice in the control group (C) received normal water and food during the whole experimental period (Suppl. Fig. 1). In contrast, after the acclimatization time of one week receiving a normal diet, mice in the PC and PC+Se groups received the metal mixture (As, Cd, Hg) in the drinking water and the drug mixture (diclofenac DCF, flumequine FLQ) in the feed for two additional weeks. Doses selection of the pollutant mixture components was based in the literature and our previous work (Fekadu et al. 2019, García-Sevillano et al. 2014b, Garcia-Sevillano et al. 2013, García-Sevillano et al. 2014a, González-Gaya et al. 2022, Huertas-Abril et al. 2023, Kashida et al. 2006, López-Pacheco et al. 2019, Parra-Martínez et al. 2022, Rodriguez-Moro et al. 2020, Trombini et al. 2021), and taking into account that the doses that humans actually receive may be significantly higher than those present in the environment due to bioaccumulation phenomena along the food chain (Chormare &Kumar 2022, Nagpal et al. 2018, Nilsen et al. 2019, Zenker et al. 2014). The estimated daily intake of As, Cd, Hg, DCF, and FLQ during the treatment period were 3.0, 0.1, 1.0, 625, and 20 mg/kg bw, respectively.

To study the potential protective effect of selenium (Se) against the toxicity of PC, one of the experimental groups (PC+Se) was fed Se-enriched chow during treatment. Selenium is incorporated into numerous human food supplements for its immunoregulatory and antioxidant properties. However, Se also has a toxic side, so its intake must be controlled and most dietary supplements for humans usually do not exceed 3 times the minimum

recommended dose (Morris &Crane 2013). Since the standard mouse diet used in our study contains 0.21 mg Se/kg, we decided to formulate the Se-enriched diet with 0.65 mg Se/kg (Raines &Sunde 2011). The estimated Se daily intake was, hence, 40 μg/kg bw for mice fed the normal diet and about 120 μg/kg bw for those fed the Se-enriched diet.

3.2. Effects of PC on mice survival and morphometric parameters

There were no deaths during acclimation. During the treatment period we found that PC administered in water (metals) and chow (pharmaceuticals) affected the survival of the mice that decreased until 83% (Fig. 1a).

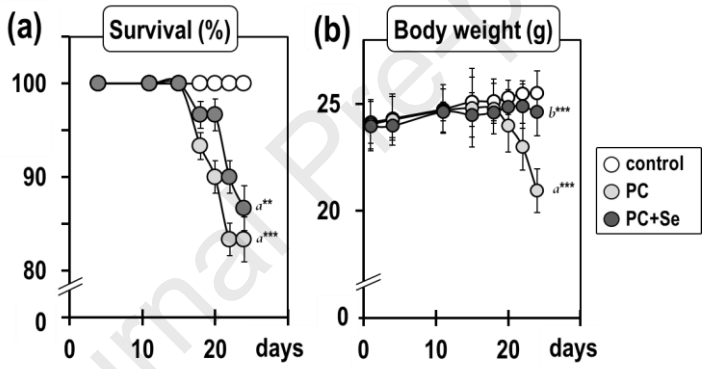

Fig. 1 Effect of exposure to PC on survival (a) and bodyweight (b). In each case, the mean ± SD is represented (n=12 mice per group). Statistical significance of the differences is expressed with letters: **a** for comparisons of control mice vs animals in the PC or PC+Se groups (Dunnett test with the Bonferroni correction); **b**, for comparison between PC and PC+Se mice (Student's *t*-test); and asterisks: * for $p<0.05$; ** for $p<0.01$ and *** for $p<0.001$.

This result was somewhat unexpected, as the doses used were lower than the individual LD_{50} values and indicated an interaction between the PC components that induced lethality. Surviving mice also exhibited a decrease in body weight of up to 20% (Fig. 1b). The impact of PC on both parameters was less pronounced when mice were fed a Se-enriched diet, which

reduced lethality and, mainly, increased body weight to values close to those of control animals (Fig. 1a, b). Metals and pharmaceutical compounds are described to alter energy metabolism and gut microbiota composition, which hinders digestion, nutrient assimilation, and weight loss (Assefa &Köhler 2020, Claus et al. 2016, den Besten et al. 2013, Di Gregorio et al. 2019, Duan et al. 2020, Elbaz et al.). Selenium participates in both processes, thus preventing the adverse impact of pollutants (Hu et al. 2018). The ability of Se to counteract the detrimental effects of certain toxic metals such as Cd or Hg and metalloids such as As is well known (Messaoudi et al. 2009, Rodríguez-Moro et al. 2020). Selenium antagonizes the effects of Cd through its ability to activate the PI3K/AKT/Bcl-2 pathway, which regulates the cellular defense system against oxidative damage, as well as cell proliferation, survival and apoptosis (Bao et al. 2017). This antioxidant capacity also permits Se to mitigate the damage caused by Hg (Fan et al. 2020) and As (Xu et al. 2013). In addition, Se binds to Hg generating biologically inactive complexes (Kuras et al. 2018) and favors As trafficking from metabolically inactive organs (brain, lung and testis) to others with higher metabolic activity (kidney), which facilitates its excretion (Rodriguez-Moro et al. 2020). The ability of Se to reduce oxidative stress (OS), inflammation and various hematological abnormalities in the liver and kidney of DCF-treated rats has also been described (Owumi &Dim 2019). These could be the cause of the lower effect of PC on the mice fed a Se-enriched diet (Fig.1a).

3.3. Histopathological analysis of liver samples

Control samples showed normal histologic architecture of the liver (Trefts et al. 2017) with moderate glycogen accumulation and clear, irregular, poorly defined spaces in the cytoplasm (Fig. 2a), as well as marginal multifocal inflammatory infiltrates normally found in the liver. In PC samples (Fig. 2b), glycogen deposits were hardly visible; instead, mild to

moderate multifocal mixed inflammatory infiltrates within the sinusoids and associated with the portal space and hematopoietic foci were observed (Fig. 2b, asterisk). Liver cells can carry out numerous separate and specialized metabolic activities, including glycogen storage. The decrease or increase in liver glycogen storage is related to diet and the health of the subject (Ulusoy &Eren 2006). The absence of glycogen deposits in PC mice livers agrees with the loss of body weight shown in Fig. 1b and indicated that the PC exposure was affecting both the liver's appearance and its functionality. The known oxidizing capacity of the components of PC may be causing this oxidative-type liver damage and may also be responsible for the inflammation shown in Fig. 2b. These PC livers additionally displayed moderate karyomegaly (Fig. 2b, arrowhead), and occasional large nuclei showing irregular shape (aberrant nuclei, Fig. 2b, arrow). The mere presence of As in PC by itself could explain the karyomegaly observed in these mice (Korany et al. 2019). Finally, the PC mice also showed some level of extramedullary hematopoiesis (EMH), considered to be the body's compensatory response to deficient bone marrow erythropoiesis or accelerated destruction of erythrocytes (Chu et al. 1999). Our findings indicate that PC is causing hematosuppression that elicit EMH as a compensatory reaction like that described for cyclophosphamide in mice (Wang et al. 2009).

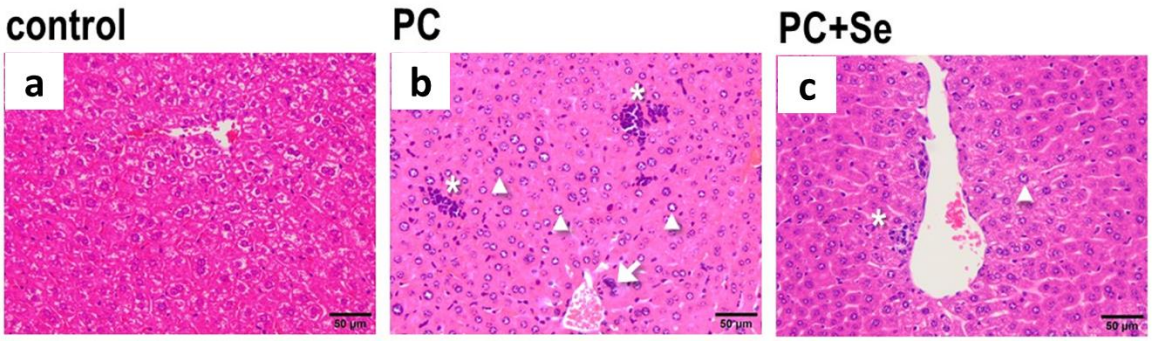

Fig. 2 Histopathological analysis of hepatic samples. A representative mouse from each group is shown. The various anomalies found are represented by asterisks (*, extramedular hematopoietic foci); arrowheads (►, karyomegaly) or arrows (→, aberrant nuclei)

The dietary Se-supplement prevented PC damage in the liver, and mice in the PC+Se group showed histologically normal livers with localized cytoplasmic glycogen accumulation around the centrilobular veins (Fig. 2c), and isolated hepatocytes with karyomegaly (Fig. 2c, arrowhead) or mononuclear-type inflammatory infiltrates (Fig. 2c, asterisk) and occasional hematopoietic foci. Hepatocytes with aberrant nuclei were not observed. These results suggest that PC exposure is causing oxidative damage to the liver, as the presence of Se, an antioxidant, protects the liver from that damage.

3.4. Plasma biochemical determinations

The results shown in Fig. 3a demonstrate that PC is causing damage to the liver tissue and the release of intrahepatic enzymes, such as AST (aspartate transaminase) and ALT (alanine transaminase), markers commonly used as clinical indicators of hepatotoxicity (McGill 2016). Consumption of a Se-enriched diet, at least partially, avoided the damage caused by PC, as the ALT and AST levels in PC+Se mice decreased and even approached control levels (AST), in agreement with the protective role of this trace element against the toxicity of pollutant mixtures described in rats (Ozardali et al. 2004).

Exposure to PC caused a remarkable increase in the bile acids (BA) concentration (Fig. 3b), suggesting alterations in their synthesis or transport. The presence of a Se-supplement in the diet of PC+Se mice partially prevented the effect of PC, confirming the ability of Se to regulate plasma BA levels (Hu et al. 2018) and to repair liver damage caused by pollutants (Morales-Prieto et al. 2018, Ozardali et al. 2004, Rodriguez-Moro et al. 2020). Total cholesterol (CHO) was not significantly affected in plasma by PC but the triglyceride (TAG) levels diminished (Fig. 3b) in agreement with the reported ability of FLQ and DCF to reduce fatty acid (FA) concentrations in hyperlipidemic rats by decreasing their export to plasma (Curcelli et al. 2008, Kang et al. 2018).

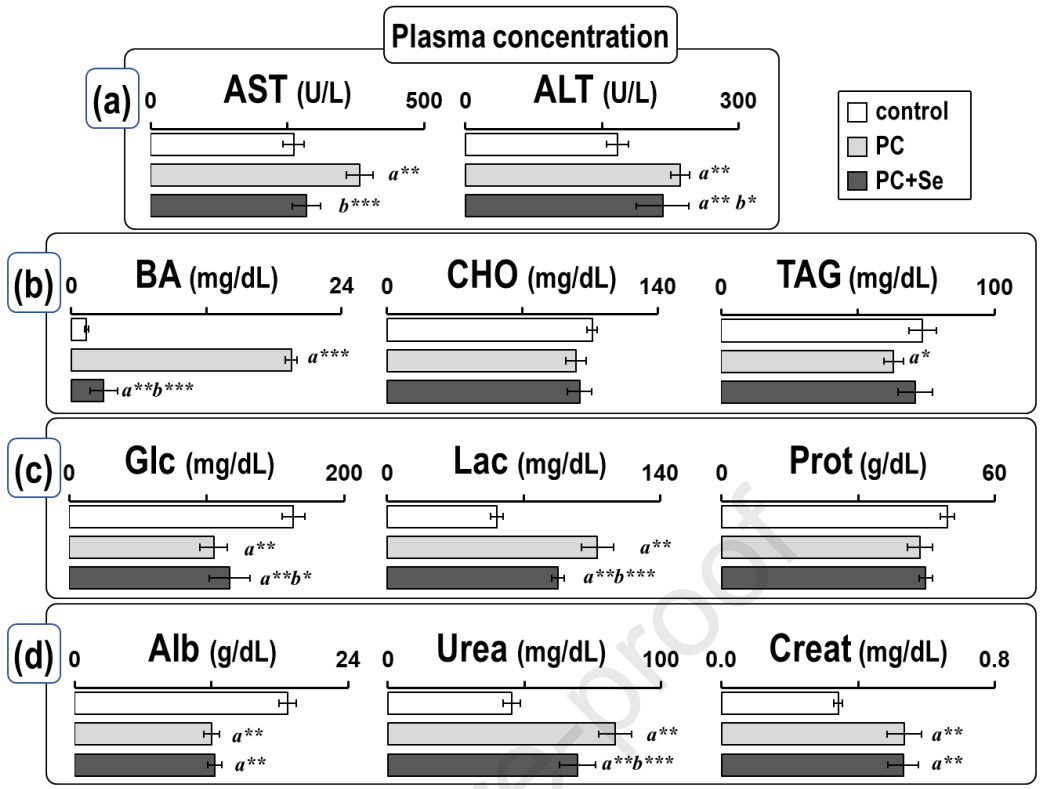

Fig. 3 Exposure to PC change several biochemical parameters in plasma. In each case, the mean ± SD (n = 12 mice per group) of three independent technical replicates is represented. Statistical significance of the differences at the end of the experimental time is expressed with letters: **a,** for comparisons of control mice vs animals in the PC or PC+Se groups (Dunnett test with the Bonferroni correction); **b**, for comparison between PC and PC+Se mice (Student's *t*-test); and asterisks: * for $p<0.05$; ** for $p<0.01$ and *** for $p<0.001$

All mice receiving the contaminant mixture (PC and PC+Se groups) showed reduced blood glucose levels accompanied by high levels of lactate (Fig. 3c). Results suggest that the combination of these contaminants or some of its components, individually or synergically, somehow affects mitochondrial functioning and its ability to generate ATP (Genchi et al. 2020, Ghosh et al. 2016, Hosseini et al. 2013, Thai et al. 2021), and forces cell metabolism into Warburg effect, *i.e.*, coupling lactic fermentation to a high rate of aerobic glycolysis (Fu et al. 2017, Gwangwa et al. 2018). Under these metabolic circumstances, glycogen is catabolized to fuel glycolysis, in agreement with the images shown in Fig. 2. This metabolic

reprogramming has been described for metals such as As (Hu et al. 2020) and drugs like DCF (Yang et al. 2021).

No significant changes in total protein levels were observed (Fig. 3c), but all animals exposed to the contaminant cocktail showed >30% reduction in albumin levels (Fig. 3d). Albumin is synthesized by the liver and rapidly excreted into the bloodstream, where it is one of the most abundant proteins. Albumin is vital for normal physiological and pharmacological responses, including the maintenance of vascular permeability and the transport of various molecules. Its high Cys-residues content gives this protein an important antioxidant function, so a reduction in its levels may act as a contributing factor in the development of several diseases related to OS, including liver disease, diabetes, and even cancer (Levitt &Levitt 2016). All components of PC have been individually associated with kidney damage (Chang &Singh 2019, Dhanvijay et al. 2013, Hazelhoff et al. 2021, Rodríguez-López et al. 2020). Data in Fig. 3d show that PC exposure elevated plasma levels of creatinine (>50%) and urea (>200%), two markers widely used in the clinic for the determination of renal damage. The presence of Se in the diet of mice partially prevented the effect of PC (Li et al. 2020).

In summary, the analysis of plasma biochemical parameters indicates that mice exposed to PC suffered hepatic and renal damage. In the case of the liver, PC exposure probably impairs the correct functioning of the respiratory chain, forcing the cell to adapt its metabolism to obtain ATP by other mechanisms (aerobic glycolysis coupled to lactate fermentation). Selenium partially counteracted the effect of PC on most biochemical parameters, which is consistent with the ability of this trace element to act as an antioxidant and to regulate metabolism directly or through modulation of gut microbiota composition (Gao et al. 2021, Parra-Martínez et al. 2022, Qiao et al. 2022, Tang et al. 2020).

3.5. Effect of PC exposure over the expression level of hepatic genes

The mechanisms of toxicity of the individual components of the pollutant mixture used in this study are complex and not fully understood to date. Numerous reports indicate that some elements (As) act by replacing phosphate in certain reactions (Hu et al. 2020); others (As, Hg, Cd) may interact with the thiol groups of proteins, inactivating them (Hu et al. 2020, Renu et al. 2021) and all of them (metals, DCF, FLQ) generate reactive oxygen species (ROS), inflammation, and various epigenetic and metabolic alterations (Kashida et al. 2006, Owumi &Dim 2019b, Renu et al. 2021).

The results shown in the previous sections suggested damages in the hepatocytes of mice exposed to PC that result in changes in glucose, lipids, and BA metabolism. To verify these hypotheses and gain deep insight into the molecular mechanisms underlying these observations, an absolute quantitative determination by qRT-PCR of some of the transcripts of genes involved in the different pathways mentioned above was performed. Gene expression profiles provide valuable information on the coordinated functioning of genes in response to different environmental, physiological, and pathological variables (Hazzalin &Mahadevan 2002). To date, the quantitative variant of the reverse transcriptase-polymerase chain reaction (qRT-PCR) is considered the gold standard for accurate, sensitive, and fast measurement of gene expression at the transcriptional level (Carter et al. 2020, Costa-Silva et al. 2017). The absolute quantitative reverse transcription polymerase chain reaction (qRT-PCR) relates the PCR signal to the transcript copy number by using a calibration curve obtained under identical conditions. This method exempts the use of reference genes, but places extreme demands on the quality of the RNA used and the amplification efficiency (Pfaffl 2006). In this work, we only employed RNAs with RIN (Schroeder et al. 2006) values higher than 8.5, with A_{260}/A_{280} ratios close to 2 and free of genomic DNA or proteins. All primers used (Suppl. Table 2) generated a single amplicon of the expected size with

efficiencies close to 100% within a range of 5-7 orders of magnitude, further demonstrating the absence of polymerase inhibitors in the samples.

3.5.1. PC modulates the antioxidant response mediated by NRF2

Since oxidative damage and/or interaction with thiol groups are involved in the mechanisms of toxicity of all PC single components, we analyzed the induction of the NRF2 antioxidant response by quantifying the expression levels of some genes included in this regulon. The transcription factor NRF2 (nuclear factor erythroid 2-related factor 2) is known to be one of the most important regulators of the cellular response to both OS and xenobiotic exposure, and recent studies have identified new functions of NRF2 including the regulation of inflammation, autophagy, metabolism or global proteostasis (He et al. 2020, Liu et al. 2022). The function of NFR2 is tightly regulated, as are the genes under its control, including proteins with antioxidant ability as metallothioneins (MT) and antioxidant enzymes such as heme oxygenase (HO-1, encoded by the *Hmox1* gene), superoxide dismutases (SOD), catalase (CAT); peroxiredoxins (PRDX), some biotransforming enzymes both phase I (*e.g.*, cytochrome P450 oxidoreductases, CYPs) and phase II (*e.g.*, glutathione S-transferases, GSTs), as well as a set of subsidiary enzymes that generate reducing power for these redox reactions, such as those involved in glutathione or NADPH biosynthesis (*e.g.*, glucose 6-phosphate dehydrogenase, G6PD) (He et al. 2020, Ma 2013). Fig. 4 shows the expression, at both the transcriptional and the activity levels, of some NRF2-regulated genes in mice exposed to PC under the different experimental conditions used here and compared with mice in the control group.

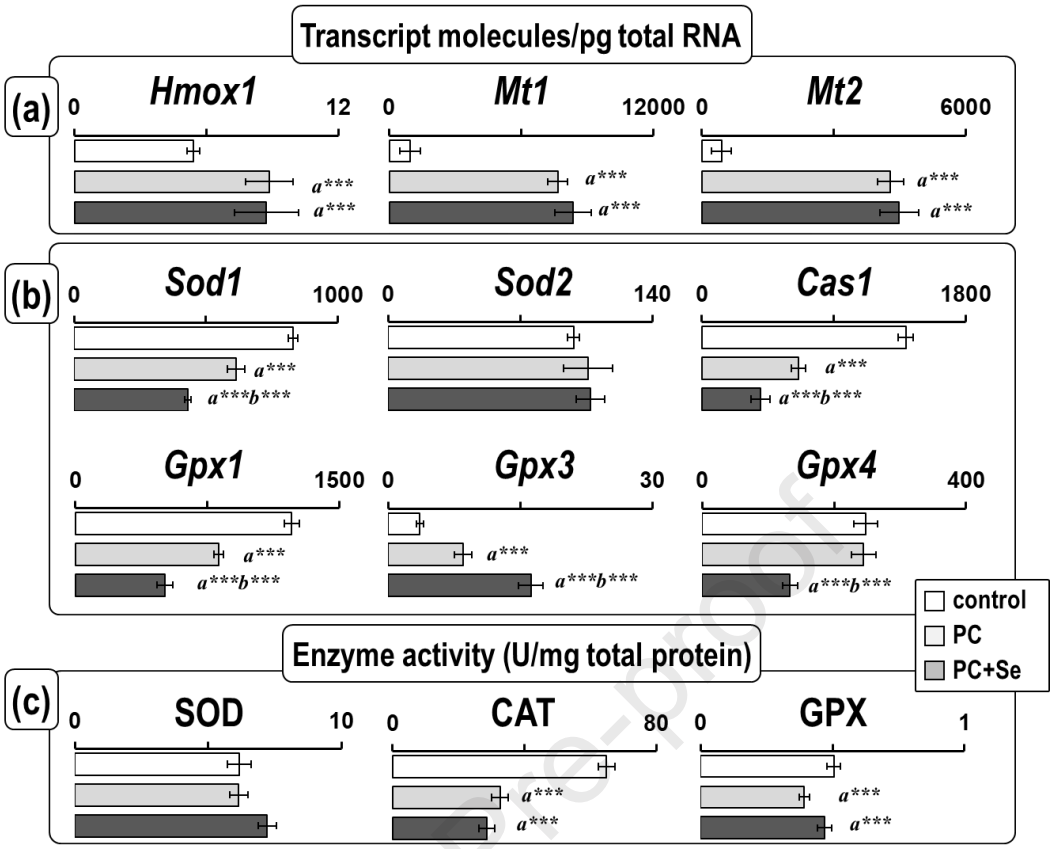

Fig. 4 Exposure to PC change the expression of several genes controlled by NRF2. See the legend in Fig. 3 for the meaning of the letters and asterisks.

Hmox1, *Mt1* and *Mt2***.** The abundance of hepatic *Hmox1*, *Mt1*, and *Mt2* transcripts increased significantly in response to PC (Fig. 4a), indicating the activation of the NRF2 antioxidative pathway. HO-1, the product of *Hmox1* gene, is a potent antioxidant enzyme regulated by NRF2 that catabolizes the degradation of the heme group released from its host proteins by oxidative protein damage and is highly cytotoxic because its iron atom may catalyze the Fenton reaction, producing free radicals, thus worsening the stress condition [reviewed in (Liu et al. 2022)]. Heme catabolism by HO-1 generates carbon monoxide, biliverdin, and free iron, each exerting, in some way, antioxidative and anti-inflammatory functions responsible for the protective properties attributed to HO-1 against OS and a wide

range of diseases, including neurodegenerative, metabolic, inflammatory diseases and cancer (Consoli et al. 2021, Ryter 2022). The increase observed for *Hmox1* transcripts is in agreement with those described in other works in which some of the contaminants used in this study were administered separately: As (Wang et al. 2019), Cd (Ashino et al. 2003), Hg (Amara et al. 2013) or DCF (Cantoni et al. 2003). Selenite has been reported to reduce (Abo El-Magd et al. 2022) or not affect the *Hmox1* expression (Wolfram et al. 2021). Accordingly, we found that the presence of a Se-supplement in the mice diet did not significantly modify the transcript levels of the *Hmox1* gene (Fig. 4a).

Metallothioneins (MT) are small, ubiquitous proteins with a high cysteine content, transcriptionally regulated by NRF2 and with an important role in the regulation of the homeostasis of essential metals (*e.g.*, Zn and Cu) and in the defense against heavy metals (*e.g.*, Cd or As) and against oxidative damage through free radical scavenging (Bensellam et al. 2021, Dai et al. 2021, Nordberg &Nordberg 2022). The *Mt1* and *Mt2* genes encode for metallothioneins 1 and 2, respectively. Both genes, highly expressed in mouse liver (Fig. 4a), with basal levels ~500-900 transcript molecules per pg of total RNA, were significantly increased (x 8-9-fold) following PC-exposure. Similar results have been described in animals exposed to metals (Chen et al. 2014, Guerrero-Castilla et al. 2014, Montes-Nieto et al. 2007) and DCF (Trombini et al. 2019, Trombini et al. 2021). Selenium has been shown to induce hepatic MT in mice and facilitate the formation of MT complexes with heavy metals, thus contributing to their detoxification (Buha et al. 2021, He et al. 2021). However, we detected only slight, non-significant increases in *Mt1* and *Mt2* transcript levels in the liver of PC+Se mice, probably because the induction of these genes by PC itself was already excessively high to be further augmented.

Sod, Cas and Gpx. The endogenous enzymatic antioxidant defense system plays an important role in protecting cells against oxidative damage. The enzymes superoxide

dismutase (SOD) and catalase (CAT, encoded by the gene *Cas1*) inactivate, respectively, superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2), transforming them into water and oxygen. Glutathione peroxidases (GPX) are also antioxidant enzymes that utilize reduced glutathione (GSH) to detoxify H_2O_2 and lipid peroxides. Induction of the NRF2 response involves the up-regulation of genes encoding these antioxidant enzymes to counteract the toxicity of reactive oxygen species (ROS) that damage biomolecules (He et al. 2020). Data in Fig. 4b demonstrate that PC exposure indeed increased the transcript numbers of *Gpx3* (coding the extracellular GPX), but did not affect *Sod2* (coding the mitochondrial SOD) and *Gpx4*, the gene coding a phospholipid hydroperoxidase protecting cells from lipid peroxidation (Flohé et al. 2022), and caused the down-regulation of the genes *Sod1*, *Cas1* and *Gpx1*, encoding the major isoforms of these enzymes in mouse liver cells. Data in Fig. 4c shown that PC exposure inhibited CAT and GPX activities, in addition to diminish their gene expression. The slight differences between the two approaches (qRT-PCR *versus* enzyme activity quantification) may be explained by the unequal effect of PC on the transcript amounts of the different isozymes, which, in contrast, were globally measured in the enzyme activity assay.

These results support our previous proteomic study (Huertas-Abril et al. 2023) and suggest that the excess of reducing equivalents resulting from continuous activation of the NRF2-antioxidative response elicited by the PC components may have led to a situation of RS in the cell (Bellezza et al. 2018, Ma et al. 2020, Perez-Torres et al. 2017, Wufuer et al. 2022). Furthermore, they demonstrated that the impairment of the antioxidant response in PC-exposed cells results from a decreased abundance of both antioxidant transcripts and proteins, as well as a reduced enzymatic activity.

Supplementation of the diet with Se somewhat diminished the inhibitory effect of PC on ~~SOD and~~ GPX activity, although it decreased the expression of *Sod1*, *Cas1*, *Gpx1* and

Gpx4 (Fig. 4b and c). The effect of the Se-enriched diet on the levels of the three analyzed GPX isoforms was, again, striking given that they are all selenoproteins whose abundance and functionality depend on the presence of Se forming part of the cysteines (SeCys) of their active sites (Minich 2022, Ye et al. 2022). However, although the underlying mechanisms are not fully understood, it is accepted a "hierarchical" regulation of selenoprotein expression by Se, resulting in "housekeeping" members (*i.e., Gpx1, Gpx4*) being held constant at the expense of "stress-regulated" members (*i.e., Gpx3*) that respond to changes in Se level (Touat-Hamici et al. 2018). Our data would confirm this Se hierarchy.

The cell redox status in the liver of PC-exposed mice. The results in Fig. 4b-c indicate that PC exposure deprives the cell of its main ROS detoxification mechanisms (*e.g.*, SOD~~1~~, CAT, GPX~~1, GPX4~~). The ability of heavy metals present in PC to bind to proteins, including transcription factors, and to impair their function is well known [*e.g.*, (Ajsuvakova et al. 2020, Shen et al. 2013)]. The observed effects could also be due to the presence of DCF in PC, as this drug has been repeatedly described to reduce the expression of these antioxidant enzymes in several aquatic animals and rats (Owumi &Dim 2019b, Trombini et al. 2019, Zhang et al. 2021), although the underlying molecular mechanisms remain to be unraveled. In the above, we suggest the possibility that the cell has entered a situation of RS attempting to mitigate the damage caused by the initiation of the antioxidant response elicited by PC exposure (Ma et al. 2020, Perez-Torres et al. 2017). This RS may occur in response to conditions in which reduced forms of important biological redox pairs, such as NAD^+/NADH, $NADP^+$ /NADPH and GSH/GSSG, predominate (Ma et al. 2020, Manford et al. 2021, Manford et al. 2020, Perez-Torres et al. 2017, Wufuer et al. 2022, Xiao &Loscalz 2020). To evaluate the redox status in the liver of PC exposed mice we quantified the malondialdehyde (TBARS/MDA) levels and the activity of the enzyme glucose 6-phosphate

dehydrogenase (G6PD) as well as the concentration of NADPH, as markers of a putative RS situation.

Lipids are primary targets of oxygen free radical attack and quantification of lipid peroxidation is commonly used to define the magnitude of OS in the cell. By using the thiobarbituric acid reactive substance (TBARS) assay, one of the most frequently used methods to assess lipid peroxidation that quantifies TBARS/MDA, we found that PC exposure reduced the oxidation state of liver cells by 41% compared to the control (Fig.5). These results reinforce the hypothesis that the cell has tried to mitigate the extreme OS elicited by the components of the PC by generating an excess of reducing equivalents driving the cell to RS (Bellezza et al. 2018, Ma et al. 2020, Manford et al. 2021, Perez-Torres et al. 2017, Wufuer et al. 2022).

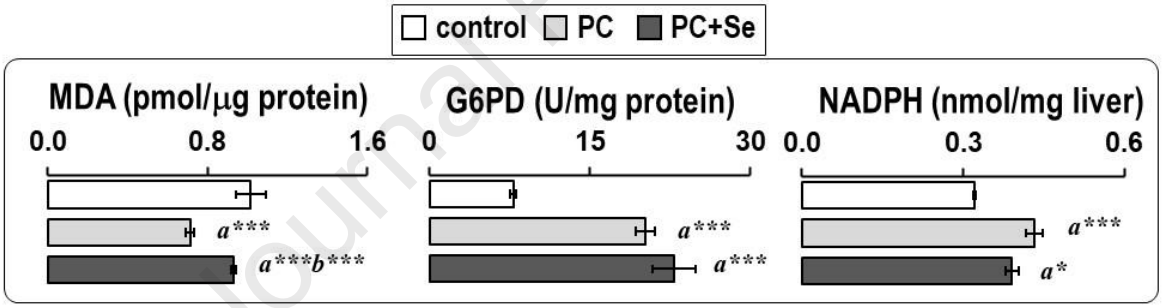

Fig. 5 Exposure to PC changes the redox state of cells in mouse liver. See the legend in Fig. 3 for the meaning of the letters and asterisks

The functioning of GPX and other antioxidant enzymes depends on the level of GSH which, in turn, depends on the presence of NADPH. Therefore, we tested the effect of PC exposure on the activity of G6PD, the first enzyme of the pentose phosphate pathway (PPP) that diverts glucose from glycolysis to NADPH generation. Figure 5 shows that all PC-exposed mice had elevated levels of this enzyme, probably signifying an excess of NADPH in the cell. To assess this point, we measured the amount of NADPH in the hepatic cells of

mice under the different experimental conditions. In agreement with the G6PD activity, we found increased levels of NADPH without changes in the level of total NADP/NADPH, in the liver of PC-exposed mice. Such an excess of reducing equivalents could have been triggered in response to oxidative stress leading to a modification of the Cys residues of the NRF2 inhibitor KEAP1. The response will deplete ROS below levels required to achieve redox signaling resulting in expected reductions in TBARS/MDA (Fig.5) (Bellezza et al. 2018, Wufuer et al. 2022). At least the metals components of the PC, Hg, As ad Cd, are proven modifiers of Cys residues in KEAP1, triggering the persistent activation of NRF2 (Buha et al. 2021, Suzuki &Yamamoto 2017) that modifies the cellular signaling pathways and the transcriptional activity, induces alterations in the formation of disulfide bonds in proteins and affects cellular metabolism (Bellezza et al. 2020, Okazaki et al. 2020). Figure 5a demonstrates the protective character of selenite, as TBARS/MDA levels were less affected in the liver of mice fed the Se-enriched diet and the NADPH levels were less deviated from basal values. The results discussed above, obtained at the transcriptional level, completely align with data of our previous proteomic study (Huertas-Abril et al. 2023) and the induction of reductive stress in hepatic cells exposed to PC.

3.5.2. Alteration of carbohydrate metabolism in the liver of PC exposed mice

Biochemical parameters shown in Fig. 3 showed reduced blood glucose levels, and high levels of lactate in all PC mice groups, which suggested cells glucose metabolism shifting from respiration to aerobic glycolysis coupled to lactic fermentation. From the results in Fig. 5, we proposed that PC exposure caused RS, compelling the cells to adopt a highly specialized metabolism favoring the synthesis of reducing equivalents that are essential for the cellular antioxidant and detoxification capacities (Okazaki et al. 2020). To study the effect of PC exposure on the glycolytic pathway we analyzed the expression of the

genes *Pfk1* (phosphofructokinase 1), the principal regulator enzyme of glycolysis that converts fructose 6-phosphate to fructose 1,6-bisphosphate; *Gapdh* (glyceraldehyde 3-phosphate dehydrogenase), which catalyzes the phosphorylation and oxidation of glyceraldehyde 3-phosphate to 1,3-bis-phosphoglycerate; and *Pk* (pyruvate kinase), the enzyme that catalyzes the last irreversible step of glycolysis, converting phosphoenolpyruvate to pyruvate. The data in Fig. 6a show that PC exposure caused an unequal effect on the expression of the three genes analyzed. *Pfk1* transcripts were significantly increased in PC mice, whereas *Gapdh* and *Pk* transcripts amount diminished.

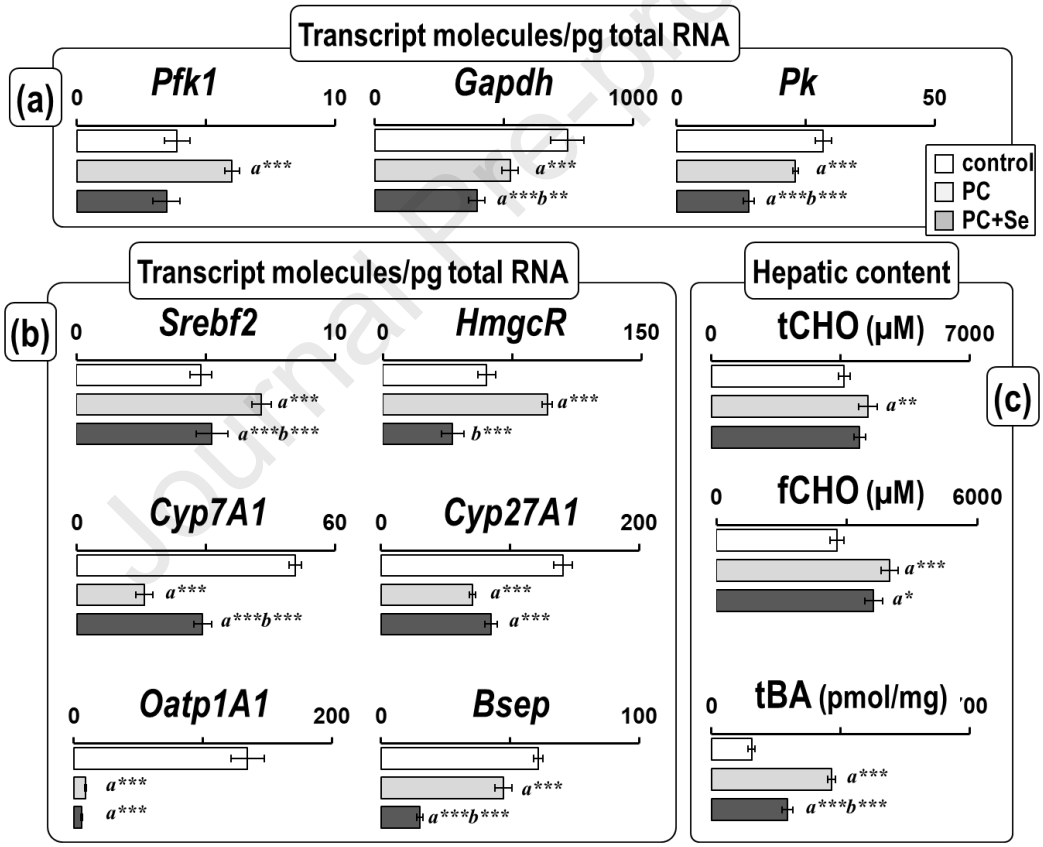

Fig. 6 Exposure to PC modifies glucose and lipid metabolism in the liver of mice. See the legend in Fig. 3 for the meaning of the letters and asterisks. Abbrev: tCHO: total cholesterol; fCHO: free cholesterol; tBA: total bile acids

The increase in *Pfk1* expression has been linked to aerobic glycolysis and its up-regulation is indicative of constitutive activation of NRF2 (He et al. 2020). In addition, PK

and GAPDH, which constitute important checkpoints of glucose flux between PPP and glycolysis, have been described to be inhibited at the gene and metabolic level in the shunting of glucose to PPP (Stincone et al. 2015). GAPDH activity reduction in PC cells would also favor the synthesis of serine/glycine from the glycolytic intermediates 3-phosphoglycerate, another factor indicative of persistent activation of NRF2 (Okazaki et al. 2020) and reductive stress generation. Se enrichment of the diet during PC exposure helped to maintain the *Pfk1* transcripts at control levels but increased the effect of PC over *Gapdh* and *Pk* (Fig. 6a). A similar effect was described in murine macrophages and would contribute to restoring cell respiration (Korwar et al. 2021).

In agreement with several recent reports (Jyothidasan et al. 2022, Shanmugam et al. 2017), our results come to show that reducing stress negatively affects some of the NRF2-regulated genes but not others additionally influenced by other compensatory transcriptional regulatory mechanism.

3.5.3. PC exposure impairs cholesterol and bile acids metabolism and trafficking in mice liver.

The analysis of the biochemical parameters (Fig. 3) indicated only small changes in the levels of CHO but increased amounts of BA in the plasma of PC-exposed mice. To evaluate how PC exposure affected the hepatic synthesis of these metabolites we quantified the expression of two cholesterogenesis-related genes, *Srebf2* and *HmgcR*, and four genes implied in the synthesis (*Cyp7A1*, *Cyp27A1*) and trafficking (*Oatp1A1, Bsep*) of BA in the liver. Data in Fig. 6b show that PC exposure caused increased transcription of *HmgcR*, the gene coding for hydroxymethyl glutaryl coenzyme A reductase that is the key enzyme in cholesterol biosynthesis, as well as of its regulator *Srebf2* (sterol regulatory element binding protein-2), a main regulator of CHO synthesis and uptake (Lefebvre &Staels 2015). The up-

regulation of both genes is consistent with the higher amount of total and free CHO detected in the liver of PC mice (Fig. 6c) and has been described as a specific feature of non-alcoholic fatty liver diseases (NAFLD) (Malhotra et al. 2020).

Cholesterol is largely converted into BA in the liver, mainly via two pathways. The canonical pathway depends on the activity of CYP7A1 (cholesterol 7α-hydroxylase the first enzyme of the pathway). The alternative, mitochondrial, pathway is mediated by CYP27A1 (sterol 27-hydroxylase encoding sterol 26-hydroxylase, the first enzyme of the mitochondrial pathway) and depends on CHO entry through the inner membrane of mitochondria (Šarenac &Mikov 2018). Gene expression analysis indicated that PC treatment inhibited both the canonical (represented by *Cyp7A1*) and the alternative (represented by *Cyp27A1*) pathways (Fig. 6b). Hence, we conclude that the increased CHO levels observed in PC mice (Fig. 6c) are caused by the sum of increased *de novo* synthesis of CHO and its reduced catabolism to BA. However, we found increased levels of BA in both the plasma (Fig. 3) and the liver (Fig. 6c) of PC-exposed mice. Bile acids newly synthetized in the liver (primary BA) are excreted, mainly as taurine and glycine conjugates, into the bile canaliculi through the bile salt export pump (BSEP) and then into the intestinal tract, where they are deconjugated and transformed by gut microbiota (GM) into new BA (secondary BA). Conjugated and unconjugated BA are reabsorbed by the intestine and effluxed to the portal circulation, which leads them back to the hepatocytes. The sodium taurocholate cotransporter polypeptide (NTCP) or the organic anion transporter (OATP) extracts the majority of BA from the blood into the liver (Katafuchi &Makishima 2022). A marked decrease in the expression levels of genes encoding the OATP and BSEP transporters was also observed in the livers of PC mice. Therefore, our data demonstrate that the high levels of BA in the plasma and the liver of these mice would be a consequence of decreased expression of both transporters. This could give rise to a situation of cholestasis, with accumulation of primary BA (synthesized in the

liver) but not secondary BA (synthesized by the GM) (Han 2018) resulting in differential regulation of the various factors involved in the synthesis of these metabolites. Besides the increase in CHO synthesis, changes in the pathways involved in the elimination of CHO were also noted in patients with non-alcoholic fatty liver disease (NAFLD). These changes included reduced BA synthesis from CHO as well as a decrease in the expression of transporters responsible for its excretion [reviewed in (Malhotra et al. 2020)]. Hence, our data indicated that PC exposure might generate NAFLD. The presence of Se in the diet reduced CHO synthesis and alleviate the accumulation of BA in the liver, so reducing the possibility of NAFLD disease, in agreement with data in the literature [*e.g.*, (Reja et al. 2020)].

4. Conclusions

The data presented here suggest that the effects on the mouse liver of exposure to mixtures of common environmental contaminants differ from those described in the literature for their individual components. Mouse survival was 83% despite the use of doses of each PC component well below their respective LD50 values. This indicates an interaction between the PC components that enhanced individual lethality. PC exposure resulted in decreased body weight of mice, suggesting changes in energy metabolism and/or composition of gut microbiota that could hinder digestion and nutrient assimilation. Altered energy metabolism may be caused by the liver damage revealed by histopathological analysis and measurement of blood biochemical parameters. And this liver damage may well be the consequence of a level of oxidative damage that would exceed the antioxidant capacity of the cells. To counteract the severe oxidative stress caused by PC, cells produce an excess of reducing equivalents. This results in reductive stress. Under this situation, the liver must adjust its metabolism to glucose fermentation because the excess of reducing equivalents

impairs the mitochondrial activity and hinder the normal trafficking of metabolites, such as cholesterol and bile acids, between organs. Given that these processes have been linked to metabolic and inflammatory disorders, as well as cancerous and neurodegenerative processes, our findings suggest that unintended exposure to these mixtures of metals and pharmaceuticals, possibly through food and water, could be the cause of numerous human health issues. Fortunately, the addition of selenium supplements at a low dose offers protective effects against the hepatotoxicity triggered by the mixture of pollutants.

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6. Statements and Declarations

6.1. Ethical approval

This work was approved by the Animal Experimentation Ethical Committee of the University of Córdoba (UCO), and by the General Direction of Agricultural and Livestock Production (Regional Government of Andalusia, Ref. 02-01-2019-001) and complied with the ARRIVE guidelines, the European Community guidelines EU Directive 2010/63/EU for animal experiments and the Spanish Government Royal Decree 1386/2018. Animal handling was conducted by qualified staff in the Experimental Animal Facility of the UCO (SAEX).

6.2. Consent to participate and publish

All authors agree with the content of the manuscript, give explicit consent to submit, obtained consent from the responsible authorities at the Universities of Cordoba and Huelva and approved the version to be published.

6.3. CRediT authorship contribution statement

PVH-A: Investigation; Formal analysis; Methodology; Data curation; Validation; Visualization; Writing-original draft; Writing-review & editing. **MJP-A:** Investigation; Formal analysis; Methodology; Data curation; Formal analysis; Validation; Visualization; Writing-original draft; Writing-review & editing. **JJ:** Formal analysis; Investigation; Writing-review & editing. **JP:** Formal analysis; Investigation; Writing-review & editing. **VM-H:** Formal analysis; Investigation; Writing-review & editing. **TG-B:** Conceptualization; Funding acquisition; Writing-review & editing. **NA:** Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Funding acquisition; Resources; Software; Supervision; Validation; Visualization; Writing-original draft; Writing-review & editing.

6.4. Fundings

Projects PG2018-096608-B-C21 and PID2021-123073NB-C21 from the Spanish Ministry of Science and Innovation (MICIN) and Regional Government of Andalusia, group BIO-187. VM-H is supported by the financial fundings of the Regional Government of Andalusia-FEDER (project P18-RTJ-1956).

6.5. Declaration of competing interest

The authors have no relevant financial or non-financial interests to disclose.

1. Figure Captions

Fig. 1 Effect of exposure to PC on survival (a) and bodyweight (b). In each case, the mean ± SD is represented. Statistical significance of the differences is expressed with letters: **a** for comparisons of control mice vs animals in the PC or PC+Se groups (Dunnett test with the Bonferroni correction); **b**, for comparison between PC and PC+Se mice (Student's *t*-test); and asterisks: * for $p<0.05$; ** for $p<0.01$ and *** for $p<0.001$.

Fig. 2 Histopathological analysis of hepatic samples. A representative mouse from each group is shown. The various anomalies found are represented by asterisks (*, extramedular hematopoietic foci); arrowheads (►, karyomegaly) or arrows (→, aberrant nuclei)

Fig. 3 Exposure to PC change several biochemical parameters in plasma. In each case, the mean ± SD of three independent technical replicates is represented. Statistical significance of the differences at the end of the experimental time is expressed with letters: **a,** for comparisons of control mice vs animals in the PC or PC+Se groups (Dunnett test with the Bonferroni correction); **b**, for comparison between PC and PC+Se mice (Student's *t*-test); and asterisks: * for $p<0.05$; ** for $p<0.01$ and *** for $p<0.001$

Fig. 4 Exposure to PC change the expression of several genes controlled by NRF2. See the legend in Fig. 3 for the meaning of the letters and asterisks

Fig. 5 Exposure to PC changes the redox state of cells in mouse liver. See the legend in Fig. 3 for the meaning of the letters and asterisks

Fig. 6 Exposure to PC modifies glucose and lipid metabolism in the liver of mice. See the legend in Fig. 3 for the meaning of the letters and asterisks. Abbrev: tCHO: total cholesterol; fCHO: free cholesterol; tBA: total bile acids

2. Supplementary Information

Supplementary Materials & Methods

1. **Experimental design and sample collection**
2. **Histopathological analyses**
3. **RNA isolation from liver samples**
4. **Primers used to quantify M. musculus liver transcripts.**
5. **Real-time qPCR amplification conditions**
6. **Enzyme activity assays.**

Supplementary Figures & Tables

Suppl. Figure 1. Experimental design

Suppl. Table 1. Chemical components of the pollutant mixture (PC)

Suppl. Table 2. Primers used for the absolute quantification of transcripts in the liver of *M. musculus.*

Highlights:

- Exposure to a metals/drugs mixture can exceed the antioxidant capacity of cells.
- Cells respond by producing excess reducing equivalents, leading to reductive stress.
- The liver switches to fermenting glucose as mitochondrial activity is impaired.
- Metabolites (cholesterol, bile acids) trafficking between organs is hindered.
- Supplementing the diet with a low dose of selenium ameliorates liver damage.