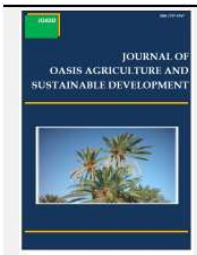

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Investigation of hepatotoxicity induced by polymetallic pollution and conventional breeding system in chickens

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Abstract

Modernization has led to environmental contamination from various chemicals, raising concerns about their impact on animal health. To investigate the specific effects of breeding methods on liver health in hens (*Gallus gallus domesticus*), focusing on two potential environmental pollution factors: heavy metal contamination and the use of veterinary additives and drugs. To achieve our purpose, the study analyzed three batches of hens: (1) controls from free-range organic farms, (2) conventional farming, and (3) free range breeding in an industrial site (Jebel Ressas old mine). After a six-month period, liver tissues were examined for the accumulation of malondialdehyde (MDA), the frequency of micronuclei (MN) as a marker for genotoxicity, and histological alterations.
The current study highlighted that varying breeding environment, potentially associated with increased use of additives, drugs, and antibiotics, notably impact the overall health of the animal liver. Moreover, exposure to heavy metals has been identified as a severe cause of health issues due to its interference with normal biological mechanisms and disruption of natural reactions. Given their non-biodegradable nature, these metals persist for extended periods, posing long-term health risks. While these findings raise concerns about the potential health risks associated with such practices. Our results suggest that exposure to heavy metals poses more serious threat. This underscores the urgent need for stricter regulations and more sustainable approaches to protect both animal welfare and public health.

1. INTRODUCTION

The annual global slaughter of chickens for meat consumption surpasses that of any other animal (Ritchie, 2020; Ahmet S.U. and Münise D., 2023). Today, more than 86 billion chickens are consumed in the world with an annual increase of 3% per year (OECD-FAO 2019). In Tunisia, statistics have shown that the consumption of poultry reaches 230 thousand tons by 2020 (Ritchie 2020). This crucial consummation has led to the development of several modes of poultry farms that aimed to intensify poultry production.

Agricultural practices, particularly intensive poultry farming, have been identified as significant contributors to oxidative stress, genetic damage, and liver dysfunction. However, the intensification of farming leads to the deterioration of the product quality (M. Estévez, 2015; Peter F. S., 2019). Veterinary drugs such as antibiotics have been extensively used in industrial chickens not only to control various diseases but also to promote growth and increase feed conversion (Youcef M. et al., 2018). Indeed, the amplification on manufacture causes the development of pathologies by the use of drugs and additives sometimes in an abusive manner (Shubhangi, 2016). In the other hand, human exposure to heavy metal causes severe health problems by affecting various organs such as liver, heart, brain and kidney. They interfere with

numerous biological mechanisms and disrupt biochemical reactions. As heavy metals are non-biodegradable, persist over time and pose long-term health risks (Hamdy, 2018).

Given the fact that the use of toxic chemicals has been increased, it is therefore appropriate to evaluate the risks incurred by the consummation of meats from animals treated by chemical molecules. In turn, efforts are increasing in order to establish methods for early assessment of the potential toxicity of these pollutants (Chardon and Burger 2014).

In this context and for environmental monitoring, avian can serve as a useful bioindicator species especially hens, which are susceptible to pollutants bioaccumulation mainly through consumption of contaminated food or being in contaminated area (Amri S., 2017). Keeping in view the above-mentioned facts, the present investigation aimed to assess the impact of different farming environments on hen (*Gallus gallus domesticus*) hepatotoxicity.

2. MATERIALS AND METHODS

2.1. Site description and animal sampling

In our study, we have compared conventional farming and free range breeding in an old mine site between two regions (Jebel Ressas and Chott Mariem) (Fig. 1).

Chott Meriem is in the northern suburbs of Sousse, Tunisia and is also known as an agricultural center. The Jebel Ressas site is located 30 km south of the city of Tunis. It is characterized by polymetallic pollution (Pb, Zn, Cd, Ni, Cu) by pointing out the presence of the old mine closed since 1956 but its waste piles are still present in the city today (Elkribi-Boukhris 2020).

For that, four batches were carried out from different sites:

- 20 hens from free range breeding (Chott Meriem) considered as control group (G1)
- 20 hens from conventional breeding from Chott Mariam region (G2)
- 20 hens from free range breeding from Jebel Ressas old mine (G3)

The analyzed hens in this study exhibited a body weight range of 780g to 930 g and were maintained under breeding conditions. After six months of breeding, the samples were immediately transported to the laboratory where they underwent euthanasia and subsequent organ analysis

2.2. Experimental analysis

Tissue dissection occurred on the day of sacrifice. The excised livers were then subdivided according to the requirements of the subsequent analyses.

2.2.1. Determination of hepato-somatic index

The Hepato-Somatic Index (HSI), which is the ratio of liver weight to body weight, was calculated from measurements taken during dissection. The formula utilized for this index is (Saif M.,2013):

HSI = (Fresh liver weight / Body weight)

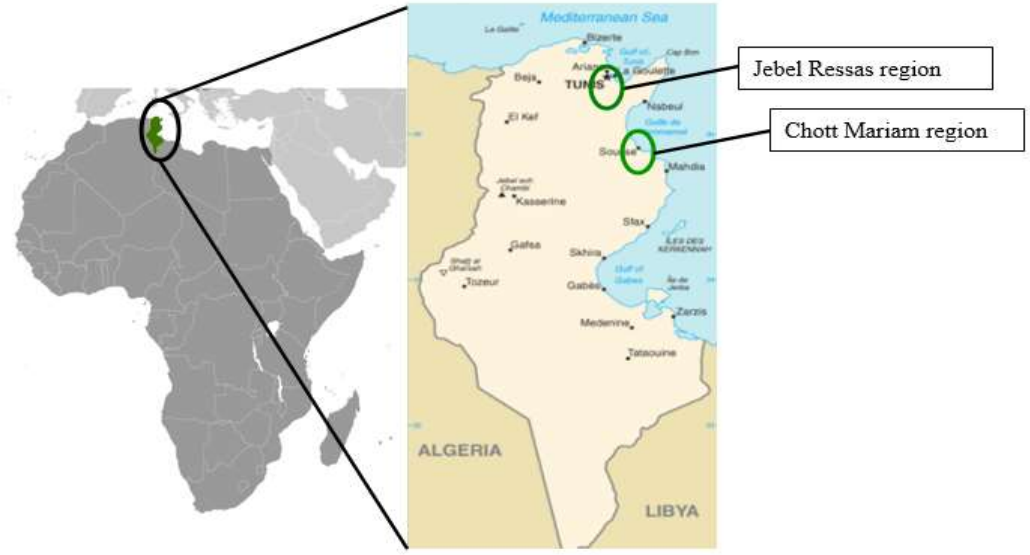

Fig. 1. Geographical location of the former mining sites of the village of Jebel Ressas and the region of Chott Mariem.

2.2.2. Determination of malondialdehydes (MDA) content

In order to make the crude extract for biochemical analysis, we start by weighing 0.1g of target tissue, then ground by the ultraturax in phosphate buffer (0.1M; pH 7.4) at a rate of 1/3 (mass/volume). After that, we centrifugate at 9000 g for 20 minutes at 4 ° C, then we distribute on several aliquots the supernatant called fraction S9, containing the microsomes and the small organelles. In order to avoid the deterioration of the enzymes, all the steps for the preparation of the cytosolic fraction were carried out at 4°C (Livingstone D.R., 2001).

We introduce respectively in a test tube:

- 800µl of trichloroacetic acid (TCA 20%)
- 2ml of thiobarbituric acid (TBA 0.67%)
- 200µl S9 fraction

Following thorough mixing, the sample was incubated in a 100°C water bath for 30 minutes. This thermal step allows the reaction of TBA with MDA, forming a characteristic pink derivative with an absorption maximum at 532 nm. The concentration of MDA, expressed in µmol per gram of tissue, was determined by the formula below:

$$\text{µmole of MDA /mg of protein} = \frac{DO532}{\varepsilon} * \text{Quantity of protein in mg}$$

With:

OD_{532}: the optical density measured at 532 nm.

ε: molar extinction coefficient of TBA (1.56.10^5).

2.2.3. Genotoxic analysis: Determination of micronuclei (MN) frequency

The MN test was performed on liver cells according to the method described by Bolognesi (1999). For that, 0.1g liver tissue are finely cut with a razor in Phosphate-Buffered Saline (PBS). Dispose with a concentration of 0.1 mg/ml previously diluted in Hanks' Balanced Salt Solution (HBSS) (2x) is added to a volume of 2 ml in the tubes containing the samples. Then a 10 min incubation of the addition of 3.4 ml of HBSS. This extract will be filtered and centrifuged at 200 g for 5 minutes at room temperature 18°C.

Aliquots of cell pellets are fixed in methanol: acetic acid (3:1) for 20 minutes, spread on slides, dried and stained with 3% Giemsa. The slides are then coded and counted.

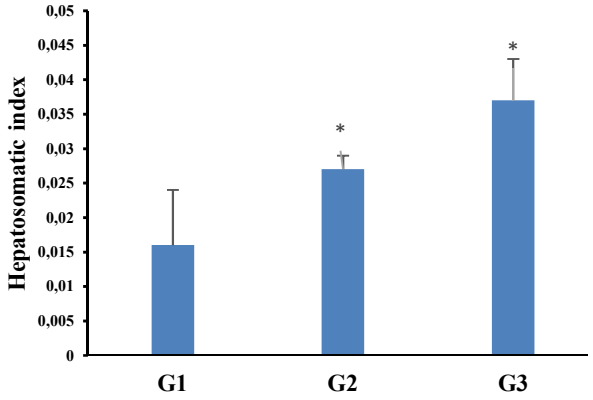

Fig. 2. Hepatosomatic index in hens from control group (G1), conventional farming group (G2), and free-range breeding in an industrial site (Jebel Ressas old mine) (G3).
**: Significant difference from control*

For each sample, two thousand cells with healthy cytoplasm are counted under oil immersion at 1000x magnification.

2.2.4. Histological analysis

For histopathological observations, liver must be fixed in order to preserve the structures and harden the tissue. Embedded in paraffin to make the section with microtome, sections was stained with hematoxylin and eosin stain. Finally, the histological images were captured using camera fitted light microscope (D. Bernet et al.,2001).

2.3. Statistical analysis

The results relating to the assay of MDA accumulation and the frequency of micronuclei are expressed as the mean ± the standard deviation (M±SD) from 20 samples. Comparison of the means is carried out by one-factor variance analysis (One WAY ANOVA) and the TUKEY test using the SPSS software (v 20.0, Microsoft), at a threshold ($P \leq 0.05$).

3. RESULTS

3.1. Determination of hepato-somatic index

Fig. 2 depicts the hepatosomatic index (HSI) values for the various groups. The data reveal significant differences in HSI among groups. The control group (G1) exhibited the lowest HSI at 0.016 ± 0.008. Conversely, hens from conventional breeding systems recorded a 1.6-fold increase in HSI compared to the control. However, the highest HSI values were observed in samples from the G3 group, which were 2.3 times greater than those from the G1 group.

3.2. Malondialdehydes accumulation

Fig. 3 illustrates the accumulation rates of malondialdehyde (MDA) in hen livers from different farming groups. The control group exhibited the lowest mean MDA levels at 0.15 ± 0.041 µmol/g of liver tissue. Hens from conventional breeding systems (G2) demonstrated a significant increase in MDA levels, reaching 0.29 ± 0.06 µmol/g. However, the highest MDA accumulations were observed in samples from the G3 farm, reaching 0.41 ± 0.10 µmol/g of liver tissue.

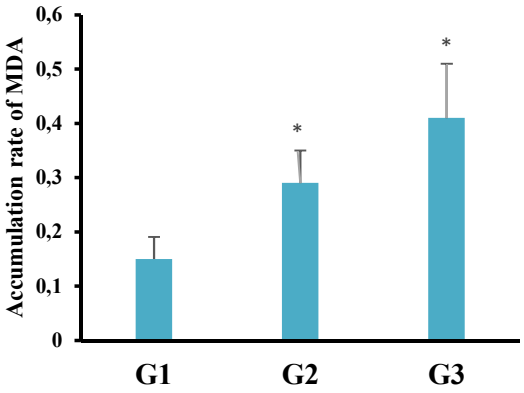

Fig. 3. Rate of accumulation of MDA expressed in µmole of MDA/g of tissues in the liver of hens from control group (G1), conventional farming group (G2), and free range breeding in an industrial site (Jebel Ressas old mine) (G3).
**: Significant difference from control*

3.3. Genotoxic results

The results of the micronucleus frequency (MN/ 1000 cells) test are presented in Fig. 4, which was used to assess genotoxic effects. The control group exhibited the lowest micronucleus frequency at 8 MN ± 1.58 per 1000 cells. Hens from conventional breeding systems demonstrated a tenfold increase in micronucleus frequency, reaching 86 MN ± 9.22 per 1000 cells. However, the highest micronucleus frequencies were observed in samples from farms exposed to heavy metal pollution, averaging 150 MN ± 16.82 per 1000 cells.

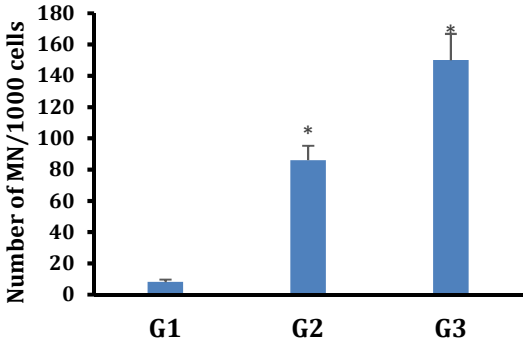

Fig. 4. Frequency of micronuclei (MN/ 1000 cells) in liver cells in hens from control group (G1), conventional farming group (G2), and free-range breeding in an industrial site (Jebel Ressas old mine) (G3).
**: Significant difference from control*

3.4. Histological results

Hens from the control group (G1) exhibited no clinical signs and displayed normal hepatocyte arrangement (Fig. 5A). In contrast, hens from the intensive (G2) and heavy metal contaminated (G3) groups revealed two primary types of hepatic lesions: inflammatory lymphocyte infiltration around blood vessels and/or portal spaces (Fig. 5B, C, D, and E). The severity of these tissue alterations varied across breeding groups. Specifically, the intensive breeding group (G2) displayed liver alterations in 25% of hens (Fig. 6). However, hens from the contaminated group (G3) exhibited a significantly higher rate of lesions, with 80% of examined subjects showing evidence of inflammatory lymphocyte infiltration around blood vessels and portal spaces.

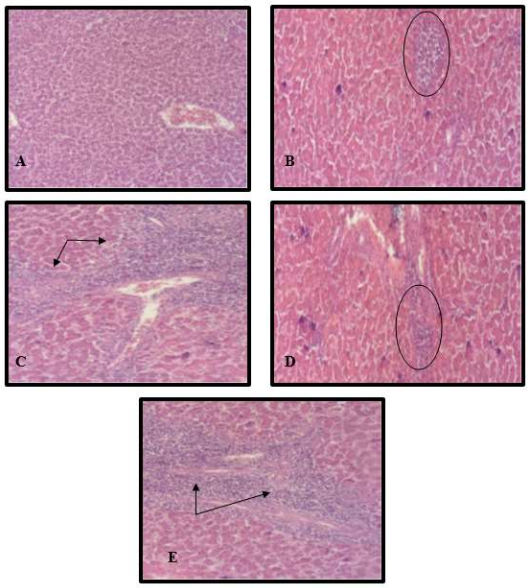

Fig 5. Hematoxylin & Eosin stained liver: (A) Normal-looking liver (G1 group): Homogeneous hepatocytes arranged in regular rows. (C and E) Presence of an inflammatory lymphocyte infiltrate around the blood vessel (arrow). (B and D) Abundant lymphocytic inflammatory infiltrate around the portal space (circle).

4. DISCUSSION

Given the increase of consumer demand for agricultural products, more particularly, those from livestock, especially the poultry industry

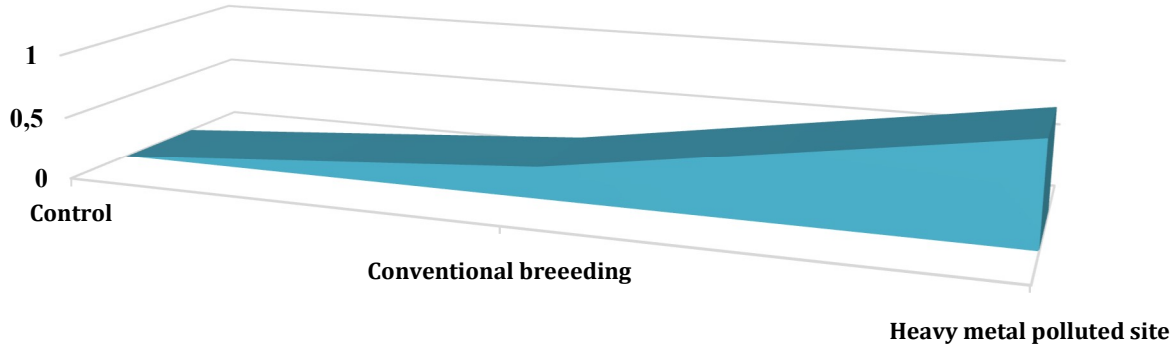

Fig. 6. Frequency of histopathological alterations depending on method of breeding: hens from control group (G1), conventional farming group (G2), and free-range breeding in an industrial site (Jebel Ressas old mine) (G3).

that has seen the most significant advancements in intensive production methods. This imposes the use of chemicals such as antibiotics and additives causing alterations and toxicity to the organisms and more specifically an important risk of xenobiotics transmission to the final consumer via food chain , thus, engendering a public health problem (Chardon and Burger, 2014; Rezaei, 2016; Elkribi Boukhris, 2021).

In the other hand, heavy metal exposure has been linked to a range of severe health problems due to their ability to disrupt normal biological processes. These non-biodegradable pollutants persist in the environment, posing a long-term threat to human health (Hamdy, 2018).

The risks that threaten the consumer have created an urgent need to develop chemical and biological analytical methods in order to follow the accumulation of toxic elements along the food chain (Kumar, 2020; MU Rehman U., 2021; Okeke E.S., 2022). On other hand, biomonitoring studies have mainly concerned coastal areas and have used several marine bioindicator species, such as the mussel *Mytilus galloprovincialis* and the sea worm *Hediste diversicolore* (Missawi, 2021; Romdhani, 2022).

Pioneering the use of hens as bioindicators, this research investigates the potential to predict the outcomes of various farming environment and rearing conditions. In order to achieve this objective, we employed a comprehensive suite of biochemical, genotoxic and histological biomarkers, constituting an index allowing a better understanding of the animal state in order to evaluate the impact of polymetallic contamination as well as the veterinary additives on hen's hepatotoxicity.

To begin with the hepato-somatic index (HSI) is an interesting indicator of the general state of the organism. Indeed, a low HSI reflects low hepatic stress while an increase in HSI can be detected during contamination by a toxicity alert (Grinwis, 2000). Nevertheless, the HSI can also undergo seasonal variations related to reproduction (Kleinkauf 2004). The result obtained for HSI in hens from intensive production is in agreement with the research of Dupuy (2012) and Cardoso (2015), which revealed that veterinary drugs could damage the liver by rising organ weight and increasing fat content and cell necrosis associated with fibrosis, which is in agreement with the results of Iskandar (2023) who proved that fish from intensive breeding generally show an increase of the HSI.

On the other hand, regarding results from the old mine site (G3) revealed the highest rates of HSI due to the important levels exhibited by heavy metal contamination in this area. This initial index reveals the potential for heavy metal pollution to be as damaging, if not more, than medical additives, which is more detailed and explained by the subsequent analyses.

Malondialdehyde (MDA), a product of lipid peroxidation, serves as a reliable marker of oxidative damage (Jebali, 2011). Our study reveals elevated MDA levels in hens from the G3 group. This increase aligns with the observed rise in oxidative degradation of polyunsaturated fatty acids within the membranes of their hepatocyte cells. Furthermore, the significant rise in MDA levels in hens from Jebel Ressas aligns with

previous research by Elkribi-Boukhris (2020) and Boughattas (2016) who observed similar increases in MDA levels in hen's kidneys and earthworms, respectively. Attig (2014) further demonstrated a positive correlation between MDA production and exposure to heavy metal pollution in mussels. Similarly, our study observed elevated MDA levels in hens raised in intensive breeding systems compared to those from traditional and organic farms. This increase likely stems from contamination by various drugs and chemicals commonly used in industrial poultry farming. Supporting this notion, Rodrigues (2016) and Wang (2018) reported elevated lipid peroxidation rates in fish exposed to antibiotics and food additives.

On the other hand, genotoxicity refers to the damage inflicted on genetic material by chemical or physical agents. Numerous studies have established a link between increased MN in cells and the development of genotoxic and cytotoxic effects (Fenech, 1999; Banni, 2009).

The highest number of MN observed in hens from the Jebel Ressas old mine farm likely results from their exposure to polymetallic pollution (Pb, Cd, Zn, and Cu). This aligns with Morcillo (2016) study demonstrating significant DNA degradation in fish exposed to various heavy metal doses. Furthermore, the significant increase in MN in hens from industrial breeding compared to control farm might be attributed to the use of antibiotics throughout their life cycle. This finding is supported by Botelho (2015) research, which observed genotoxicity in fish exposed to antibiotics during their lifespan.

Conversely, histopathological changes, often irreversible consequences of sublethal stressors, serve as valuable tools for evaluating contaminant toxicity (Lam & Gris, 2003). Numerous studies report histopathological changes in earthworms exposed to organic pollutants and heavy metals (Lourenço, 2011). Our findings revealed a significant contamination rate (80%) in animals from the Jebel Ressas site compared to all analyzed samples. These animals exhibited inflammatory lymphocyte infiltration around blood vessels and/or in the portal space. Similar damage was observed in Eisenia andrei earthworms following metal contamination (Lourenço, 2011), suggesting a link between the observed histological changes and exposure to metals and radionuclides in the soil.

Furthermore, cytotoxicity and histological results appear to be concordant, implying that tissue damage likely follows cellular alterations. Histology examines the morphological and structural changes at the tissue level, such as necrosis, inflammation, vacuolization, or fibrosis. The concordance implies that the early cellular stress observed in the cytotoxicity assays progresses into the visible, macroscopic tissue damage confirmed by histology. This strengthens the overall conclusion by demonstrating a logical cascade of injury from the cell to the organ. Consistent with findings in other animals, our study suggests that breeding conditions, particularly exposure to heavy metals and/or veterinary drugs, induce hepatotoxicity in hens.

Our findings suggest also that polymetallic contamination exerts a more significant impact on liver damage compared to the combined effects of food additives and veterinary drugs commonly used in intensive livestock farming.

5. CONCLUSION

This study investigated oxidative stress, cellular and DNA damage in the liver tissues of chickens raised for six months in different environments. The goal was to assess hepatotoxicity based on rearing conditions. Our findings revealed extensive damage at both the cellular and tissue levels. This included increased levels of MDA and HIS, elevated micronuclei (MN) frequency, and the presence of inflammation observed through histological analysis.

These observations highlight the detrimental effects of chronic exposure to a polymetallic-polluted environment and the potential hepatotoxicity of drugs used in intensive farming practices throughout the animals' life cycle. While findings suggest potential toxicity from heavy metal exposure followed by veterinary additives and antibiotics, it is important to note that both rearing environments contribute to liver damage at the cellular and tissue levels. However, our results indicate that polymetallic contamination appears to be a more significant contributor to this damage.

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Author contribution

EBS Data curation, Methodology, Formal analysis, Investigation, Supervision, Writing –original draft, Writing – review & editing. BI Formal analysis, Methodology, review & editing. HS

Methodology, review & editing. MM Data curation. BM Funding acquisition, Resources, review, Validation.

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