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# Beyond visual phytotoxicity: A standardized in vivo evaluation of cumulative systemic damage from potentially toxic elements (PTEs) in sewage irrigated crops.

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# Abstract

Wastewater irrigation facilitates the entry of potentially toxic elements (PTEs) into the food chain, often posing significant health risks even when crops appear visually healthy and phytotoxicity symptoms are absent. This study investigated the sub-chronic toxicological impacts of consuming heavy metal-contaminated spinach and cabbage grown in sewage-irrigated soils from three industrial districts in Pakistan (Chiniot, Faisalabad, and Kasur) using a Wistar rat model. Rats were fed a diet containing 20% contamination-derived crops for 90 days. Results indicated a dose-dependent increase in Bioaccumulation Index (BAI) and tissue-specific toxicity, with Cadmium levels following the descending order: liver > kidney > lungs > brain > heart. Consumption of contaminated produce caused significant ($P < 0.05$) physiological impairment, including an average reduction in body weight of 110g in the highest contamination group (Kasur) compared to the control. Hematological profiles showed an anemic condition, characterized by reduced red blood cells (RBC), hemoglobin (HGB), and hematocrit (HCT), alongside leucocytosis. Biochemical analyses revealed hepatotoxicity and nephrotoxicity, evidenced by elevated Aspartate aminotransferase (AST), Alanine amino transferase (ALT), Alkaline Phosphatase (ALP), and Creatinine (CRE), and decreased serum total protein (TP) and albumin (ALB). Furthermore, the brain exhibited the highest susceptibility to Pb attack, while bone tissues acted as the primary long-term sink for lead deposition. These findings demonstrate that visual inspection of crops is insufficient for assessing safety. The study provides a robust toxicological framework linking environmental contamination to biological endpoints, suggesting a high risk of chronic systemic collapse in human populations consuming similar produce.


# 1 Introduction

PTEs are global contaminants present everywhere in the environment. They are persistent in nature and are opportunistic in entering the food chain (Ali, Khan, & Ilahi, 2019). Wastewater irrigation is a promising resource of many important plant nutrients which can improve soil health and enhance the food quality as well. The major downside in its use is the presence of toxic metals in wastewater. The PTEs can cause serious health problems to both animals and humans who consume the contaminated food. This problem has attracted a large number of studies devoted to health risk assessment and possible intervention measures. Standards and guidelines have been set by the World Health Organization (WHO) and the US Environmental Protection Agency (USEPA) for irrigation water following the wastewater treatment. However, there is persistent risk remaining in the reuse of treated wastewater. So, it is necessary to monitor the toxic effects of PTEs in wastewater for both animals and plants on regular bases (Bartholomaeus, Parrott, Bondy, Walker, & Foods, 2013).

The threat of food contamination by PTEs has become a public health problem of world-wide concern (Abdullahi et al., 2021). Consumption of toxic metal contaminated vegetables grown in sewage irrigated lands is a major route of human contamination with these metals (Habu et al., 2021). It is common practice to use treated wastewater for irrigation purposes as it contains less risk as compared to raw city effluent. In an investigation by Dhir et al. (2015), tomato plants were grown with treated wastewater

and fed to Wister rats for 14 days. There were no abnormal changes were observed in hematological, biochemical and physical parameters. This was a short-term study and the results were not very informative. To gain a better handle on the problem, there is a need for long-term study on chronic effects of PTE exposure from irrigation with treated wastewater and is the goal of this part of my experiment (Dhir, Nasim, Nafees, & Srivastava, 2015).

The Jakara and Getsi Rivers have been continuously receiving untreated wastewater. Cabbage and lettuce cultivated on irrigation farms along these rivers were harvested and fed to albino rats over a 90-day period. Elevated concentrations of heavy metals, particularly lead (Pb) and cadmium (Cd), were detected in various body organs of the rats, surpassing levels of other toxic metals. Significant reductions in the weights of the liver, kidneys, and pancreas were observed. Cadmium accumulation was highest in the liver, heart, and kidneys, and lowest in the pancreas. Similarly, the weights of organs such as the liver, pancreas, and heart were markedly reduced. These findings suggest that consumption of contaminated crops not only leads to bioaccumulation of toxic metals but also causes organ atrophy, indicating severe metal toxicity. It is plausible that local populations consuming such contaminated produce may exhibit similar toxicological symptoms (Muktar, Alhassan, Atiku, Pedro, & Wudil, 2021).

Lead and cadmium are among the most pervasive environmental and occupational toxicants, posing significant risks to both animal and human health (Mol, 2011). Lead toxicity is associated with the generation of reactive oxygen species (ROS), induction of apoptosis, depletion of intracellular glutathione, and mitochondrial damage (Sabath & Robles-Osorio, 2012). Cadmium toxicity involves its transport to the liver via blood albumin (ALB), where it binds to metallothionein (MT) to form a Cd-MT complex, which is subsequently released into systemic circulation (Hambach et al., 2013). Co-exposure to Pb and Cd may result in synergistic toxic effects, exceeding the impact of individual metals (Wang & Fowler, 2008). Both metals share similar target organs and mechanisms of toxicity, including excessive ROS production and inhibition of sulfhydryl-containing enzymes (Dai et al., 2013; Hambach et al., 2013).

Early symptoms of potentially toxic element (PTE) exposure may include headaches and general weakness. However, accurate diagnosis requires clinical expertise in metal toxicology and appropriate diagnostic testing (Hu, 2002; Järup, 2003; Wallace, 2015). The severity of health outcomes is directly influenced by the concentration, duration, and chemical form of metal exposure. Toxic metals compromise human health primarily through oxidative stress, leading to ROS generation, enzyme inactivation, and suppression of antioxidant defense mechanisms (Balali-Mood, Naseri, Tahergorabi, Khazdair, & Sadeghi, 2021; Navas-Acien et al., 2005; Prozialeck, Edwards, & Woods, 2006).

Both acute and chronic exposure to heavy metals have been linked to a range of behavioral and physiological disturbances in humans and animals. These effects can culminate in severe health complications, including carcinogenesis and mortality (Navas-Acien et al., 2005; Prozialeck et al., 2006). At the cellular level, metals interfere with proliferation, growth, differentiation, and apoptosis (Balali-Mood et al., 2021). Clinical manifestations of metal poisoning include neurobehavioral disorders,

intellectual disabilities in children, renal dysfunction, dementia in adults, hepatic impairment, depression, visual disturbances, insomnia, and emotional instability(Jan, Ali, & Haq, 2011).

The selection of Chiniot, Faisalabad, and Kasur as study sites provides a representative gradient of the pollution crisis in Punjab's agricultural heartland. According to Quddoos et al. (2024), Faisalabad, characterized by its massive textile and chemical industry, discharges untreated effluents into drains subsequently tapped for irrigation, a practice that(Quddoos, Muhmood, Naz, Aslam, & Usman, 2024) identified as a major driver of food chain contamination. In contrast, Kasur represents a unique toxicological profile due to its globally recognized tanning industry. Recent geospatial assessments by Gulfam, 2021 have confirmed that the tannery-dominated soils of Kasur harbor high concentrations of bioavailable Chromium and Cadmium, creating a toxic "hotspot"(Gulfam, 2021). Meanwhile, Chiniot, though traditionally less industrialized, is increasingly becoming an area of concern as PTEs footprints expand across the Indus Basin (Aftab et al., 2023). By comparing these three districts, this study highlights how varying degrees of industrialization and sewage-irrigation practices directly translate into differential bioaccumulation rates and systemic health risks for the local population. Unlike previous studies that rely primarily on mathematical health risk assessment (HRA) models and theoretical indices to estimate potential hazards, this research employs a direct *in vivo* mammalian model (Wistar rats).

## 2 Materials and Methods

# 2.1 Location of the study

The study was conducted at the Institute of Animal and Dairy Sciences, Faculty of Animal Husbandry, University of Agriculture Faisalabad, Pakistan.

# 2.2 Animal selection

Albino rats were purchased from Institute of Animal and Dairy Sciences, Faculty of Animal Husbandry, University of Agriculture Faisalabad. 12 healthy male rats weighing 115g to 120g approximately were selected for this study.

# 2.3 Animal care and ethical standards

The animals were kept at standard laboratory conditions. They were provided 12 hours light/dark cycle in a day. Suitable temperature and relative humidity conditions were provided i. e., 25 ± 3C° and 40 ± 5% respectively. The study was conducted with the permission of the Institutional Biosafety and Bioethics Committee, University of Agriculture Faisalabad in order to follow all the ethical standards and experimental procedures prevalent for animal care internationally (Approval No: 1277/ORIC/28-2-2023).

# 2.4 Animals diet selection

To probe the actual impacts of sewage irrigated diet on living systems, the sewage irrigated crops were collected from three districts of Punjab including Chiniot, Faisalabad and Kasur as many of the previous studies conducted by different researchers have reported ground water, soil and crop contamination with

toxic metals. Hyper accumulative, sewage irrigated crops such as spinach and cabbage were obtained from these districts. Crops were washed with deionized water to remove surface dust, air-dried, and then oven-dried at 70°C until a constant weight was achieved. The basal diet was formulated according to the AIN-93G standard for growth(Griffin, Radhakrishnan, & Pellizzon, 2022). The diet provided approximately 19% protein, 7% fat, and 5% fiber, ensuring optimal nutritional support during the 90-day trial. To investigate PTEs toxicity, the treatment diets were prepared by incorporating 20% (w/w) dried biomass each of contaminated spinach and cabbage into this AIN-93G matrix. The inclusion of the biomass was balanced against the carbohydrate component to ensure all diets remained isocaloric and isonitrogenous, thereby isolating the effects of metal toxicity from nutritional variables.

# 2.5. Experimental Design and Procedure

Wistar Albino rats were selected as the mammalian model due to their physiological and metabolic similarities to humans, allowing for the extrapolation of potentially toxic element (PTE) effects on human health. A total of 12 rats were randomly assigned to four experimental groups (n = 3) per group).

Prior to the trial, animals were acclimatized for 15 days under standard laboratory conditions and provided a basal AIN-93G purified diet with water *ad libitum* obtained from the reverse osmosis (R.O) plant of the University of Agriculture, Faisalabad.

Following acclimatization, a 90-day sub-chronic feeding trial was conducted using the following dietary regimens:

- **Group 1 (Control)**: Basal AIN-93G diet supplemented with 20% (w/w) spinach and 20% (w/w) cabbage powder cultivated with canal water.
- **Group 2 (Chiniot)**: Basal diet supplemented with 40% (w/w) composite vegetable powder (spinach + cabbage) harvested from sewage-irrigated sites in Chiniot.
- **Group 3 (Faisalabad)**: Basal diet supplemented with 40% (w/w) composite vegetable powder harvested from sewage-irrigated sites in Faisalabad.
- **Group 4 (Kasur)**: Basal diet supplemented with 40% (w/w) composite vegetable powder harvested from sewage-irrigated sites in Kasur.

The vegetable biomass was dehydrated at 60°C and finely ground before being incorporated into the purified AIN-93G matrix to ensure an isocaloric and isonitrogenous diet across all groups.

# 2.6. Animal Behavior Assessment

To evaluate the neurobehavioral impact of lead (Pb) and cadmium (Cd) in the absence of specialized automated tracking systems, a systematic Functional Observational Battery (FOB) was implemented. This protocol focused on visually detectable physiological and behavioral markers of neurotoxicity, including the presence of porphyrin staining ('red tears'), piloerection, and kyphosis (hunched posture) as indicators of systemic distress and skeletal discomfort(Debnath et al., 2025). Motor coordination and sensory-motor integration were qualitatively evaluated through manual assessments of the righting

reflex, gait analysis for ataxia, and wire-lid grip strength. Additionally, emotional reactivity and anxiety-like behaviors manifesting as increased irritability during handling and thigmotaxis (wall-hugging) were recorded to monitor the disruption of limbic and cortical pathways typically associated with heavy metal accumulation (Mukhi et al., 2024).

## 2.7. Blood Collection and Processing

Upon completion of the 90-day exposure period, rats were subjected to mild inhalation anesthesia. Approximately 4–5 mL of blood was collected via cardiac puncture using heparinized syringes. The blood was divided into three distinct aliquots: (i) for immediate hematological profiling, (ii) for serum biochemical assays (maintained at 4°C during transport), and (iii) stored at -20°C for subsequent PTE (Cd and Pb) quantification.

## 2.8. Tissue and Bone Sampling

Following blood collection, animals were fasted overnight, weighed, and humanely sacrificed. Vital organs (liver, kidneys, lungs, heart, and brain) were meticulously excised, rinsed with 70% ethanol and deionized water to remove exogenous contaminants, and weighed using a high-precision electronic balance (± 0.001g). The entire carcass (after removal of fat and internal organs) was collected and stored at -20°C for subsequent toxicological evaluation. For skeletal analysis, femurs were removed, air-dried, and subsequently oven-dried at 80°C until constant weight. The dried bones were pulverized into a fine powder for trace element analysis.

## 2.9. Monitoring Body and Organ Weight Dynamics

To monitor the progression of systemic toxicity, animal body weights were recorded tri-weekly throughout the 90-day duration. The organ weight was measured at the end of the experiment immediately after the dissection of rats. The rats were overnight fasted before they sacrificed. The treatment groups were compared to the control group to determine the organ atrophy and weight loss among the treated rats.

**2.10. Bioaccumulation Index (BAI)** The Bioaccumulation Index (BAI) for various organs was calculated to quantify the efficiency of metal transfer from the diet to the mammalian tissues. The index was determined using the formula proposed by Nawrot et al. (2010).

$$\mathrm{BAI} = \frac{\mathrm{Ma} - \mathrm{Mb}}{\mathrm{Mb}}$$

Where Mb is the concentration of metal in control group rats and Ma is the concentration of metal in treated group rats exposure (Nawrot et al., 2010).

## 2.11. Sample Digestion for PTE Analysis

For metal quantification, 500 mg of wet tissue (or bone powder) and 1 mL of blood were subjected to wet acid digestion. Samples were treated with a di-acid mixture ($HNO_3$:$HClO_4$) in a 4:1 ratio, analytical grade) and allowed to pre-digest overnight at room temperature to ensure complete breakdown of organic matter. The mixture was then heated on a hot plate at 100 C for approximately 45 minutes until the volume was reduced to ~ 1 mL and the digestate became clear. The solution was diluted to 100 mL with deionized water and filtered through Whatman No. 42 filter paper before analysis via Atomic Absorption Spectroscopy (AAS).

# 2.12. Instrumental Quantification and Quality Control

The concentrations of Pb and Cd were determined using a Thermo S-Series Atomic Absorption Spectrophotometer (USA). The instrument was calibrated using multi-element standard stock solutions (1000 ppm, Merck). Quality control was maintained by analyzing reagent blanks and spiked samples in triplicate. Precision and recovery rates were validated against Certified Reference Materials (CRM), and instrumental parameters were optimized as per Shehzad et al. (2019) displayed in Table 2.1 (Shehzad et al., 2019).

Table 2.1
Parameters of AAS

| PTEs | Units | Detection limit | Certified value | observed value | Accuracy error (%) | Recovery (%) |
|---|---|---|---|---|---|---|
| Cd | mg $kg^{-1}$ | 0.003 | 54 ± 0.5 | 52.2 | 3.46 | 96.5 |
| Pb | | 0.016 | 0.140 ± 0.001 | 0.139 | 0.91 | 99.1 |

# 2.13. Hematological Profiling

Whole blood samples collected in heparinized vials were immediately transported at 4 C to the Institute of Animal and Dairy Sciences, UAF. Automated hematological analysis was performed to determine:

# 2.13.1 Erythroid indices

Red Blood Cell (RBC) count, Hemoglobin (HGB), Hematocrit (HCT), Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH), and MCHC.

# 2.13.2 Immune and Clotting markers

White Blood Cell (WBC) count and Platelet (PLT) count.

# 2.14 Biochemical assay

The blood serum was collected and different enzymatic activities including ALT and AST were assessed according to the protocols established by International Federation of Clinical Chemistry; ALP was evaluated by following the methods of German Clinical Chemistry. Similarly, Creatinine was tested by following the Jaffe method.

## 2.14.1 Alanine aminotransferase

ALT was assessed by using the commercial kit of Merck having a catalog no. 5.17531.

in the blood serum of rats.

Reagents:

R1= (L-Alanine 550 m. mol /L, Tris Buffer, pH 7, 110 m. mol/L)

R2= (α - Ketoglutarate > 13 m. mol/L, LDH = 4000 u/l, MDH = 800 u/l, NADH = 0.2 m. mol/L)

The above reagents R1 and R2 were mixed in a ratio of 4:1 to prepare a reaction solution. This solution (1mL) was taken in the cuvette following the addition of 100µl of blood. After an interval of 60 seconds three times absorbance was measured. The mean change in absorbance was measured as ΔA nm/min, and the calculation of ALT activity were made by the following formula.

ALT activity [u/l] = (ΔA nm/min) × 1746

## 2.14.2 Aspartate aminotransferase

AST was assessed by using the commercial kit of Merck having a catalog no. 5.17521.

in the blood serum of rats.

Reagents:

R1= (1. L-Alanine 264 m. mol /L, 2. Tris Buffer, pH 7.8, 88 m. mol/L)

R2= (1. α - Ketoglutarate > 13 m. mol/L, 2. LDH = 4000 u/l, 3. MDH = 800 u/l, 4. NADH = 0.2 m. mol/L)

Procedure:

The above reagents R1 and R2 were mixed in a ratio of 4:1 to prepare a reaction solution. This solution (1mL) was taken in the cuvette following the addition of 100µl of blood. After an interval of 60 seconds three times absorbance was measured. The mean change in absorbance was measured as ΔA nm/min, and the calculation of AST activity were made by the following formula.

AST activity [u/l] = (ΔA nm/min) × 1746

## 2.14.3 Alkaline Phosphatase

ALP was assessed by using the commercial kit of Merck having a catalog no. 5.17641.

in the blood serum of rats.

Reagents:

R1= (1. Magnesium chloride 0.625 m. mol /L, 2. Diethanolamine, pH 10.2, 88 m. mol/L)

R2= (p-Nitrophenyl phosphate 50 m. mol/L)

Procedure:

The above reagents R1 and R2 were mixed in a ratio of 4:1 to prepare a reaction solution. This solution (1mL) was taken in the cuvette following the addition of 20µl of blood. After an interval of 60 seconds three times absorbance was measured. The mean change in absorbance was measured as ΔA nm/min, and the calculation of ALP activity were made by the following formula.

ALP activity [u/l] = (ΔA nm/min) × 2750

## 2.14.4 Creatinine

Creatinine in the serum was evaluated by a commercially available kit of Merk having a catalog #5.17551.

Reagents:

R1= (Picric acid 8.73 m. mol)

R2= (1. p-Nitrophenyl phosphate 312.5 m. mol/L, 2. Disodium phosphate 12.5 m. mol)

Standard= Creatinine $2gdL^{-1}$

Procedure

The reagents R1 and R2 were mixed up in a ratio of 1:1 to form the reaction mixture. One ml of this reaction mixture is taken into the cuvette following addition of 100 µl of blood serum and the solution formed named as sample testing solution (As). Similarly, 1ml of reaction solution is mixed up with the standard solution which is called standard testing solution. Firstly, measured the absorbance of sample testing solution twice with a gap of one minute i.e., As1 and As2. Secondly, the absorbance of standard testing solution was measured twice as Ast1 and Ast2. The creatinine level in the serum was calculated with the help of following formula.

Creatinine in serum $(mg\ dl^{-1})$ = As2 -As1/Ast2 -Ast1 × 2

# 3 Results and Discussion

### 3.1 Clinical examination and survival of the animals:

The rats were clinically observed on daily bases to see any abnormalities in their behaviors and physique. A number of abnormal behaviors were recorded and however, no death was observed during the study time period. The exposure to Pb and Cd resulted in significant, visually observable behavioral deviations consistent with established models of heavy metal neurotoxicity. Specifically, an increase in thigmotaxis, heightened irritability, porphyrin staining and prevalence of ataxia were observed among the treated groups.

This increase in thigmotaxis and heightened irritability suggest a disruption of the animal's emotional regulation, likely stemming from metal-induced oxidative stress in the limbic system (Mfem & Oyama, 2021) These emotional shifts were accompanied by physical markers of distress, most notably porphyrin staining and a weakened grip strength, which serve as non-instrumented indicators of peripheral neuropathy and systemic physiological strain(Eskut & Koskderelioglu, 2021) The prevalence of ataxia and a delayed righting reflex further confirm the impact of these toxic metals on the cerebellum and vestibular function, highlighting the utility of qualitative observational batteries in detecting profound neurological impairment (Taba, 2014).

### 3.2 Body and organ weight dynamics:

Average decreases in body weight of 12, 38 and 110 g were observed in the three treatment groups of Chiniot, Faisalabad and Kasur respectively relative to control group rats which were consuming canal water irrigated crops. The minimum decrease in body weight was observed in Chiniot group while maximum weight loss was seen in Kasur group. Body weight changes on weekly basis are given in Fig 3.1. Similar pattern of decrease in weight was observed in vital organs. The liver weights of Chiniot group, Fsd group and Kasur group rats decreased 0.89, 2.22 and 3 g respectively as compare to the control group. The decreases in kidney weights were 0.35, 0.52 and 0.65 g respectively in Chiniot, Fsd and Kasur groups respectively in comparison with the control group. Decreases in average weights of lungs were 0.39, 0.57 and 1.27 g, brain weights were 0.22, 0.32 and 0.44 g, and heart weights were 0.27, 0.39 and 0.53 g respectively for Chiniot, Fsd and Kasur diet groups. The maximum decrease in body weights as well as in organ weights was recorded in rats of Kasur group diet. The sewage irrigated spinach and cabbage obtained from district Kasur having highest concentrations of both Cd and Pb among all other treatments were fed to these rats. There was a significant difference observed when $P \leq 0.05$ in body weight and organ weight between control group and the Kasur sewage irrigated diet groups. These results have been presented in Fig. 3.1.

The result of current study was in line with Muktar et al (2021) as they stated that the level of metal including Pb and Cd was high in different body parts as compared to the other toxic metals. A significant decrease in body weight of liver, kidney and pancreas was observed. Similarly, weight of body organs including liver, pancreas and heart was also significantly decreased. In current study the decreasing order of organ weight is Liver > lungs > kidneys > heart > brain. So the maximum weight loss was

observed in liver (Krishnan, Lui, Jervis, & Harrison, 1990).  while minimum in brain (Keiko & Minoru, 1991; Tang et al., 2016).

The consumption of these contaminated food crops not only accumulated at higher concentration in animals but also reducing the organ weights of affected rats indicated critical signs of metal toxicity for living systems (Fig 3.1 and Fig. 3.2). The local population may also exhibits similar symptoms of toxicity by consuming the contaminated food crops grown with wastewater (Muktar et al., 2021). Our results imply that the local population in Faisalabad and Kasur may be suffering from "hidden hunger" a state where caloric intake is sufficient, but systemic PTE toxicity is causing internal organ atrophy and metabolic decline. This was a sub-chronic (90-day) study. While this is sufficient to show initial organ damage, it may not fully capture the cumulative "bio-magnification" that occurs over a human lifetime (20+ years) of consumption.

### 3.3. Distribution and Bioaccumulation of Cadmium in Rat Tissues

Analysis of tissue distribution revealed that cadmium (Cd) concentration was significantly higher ($P < 0.05$) in the blood and muscular tissues of rats in Group 4 (Kasur diet) compared to the other treatment groups. The highest Cd concentrations were recorded in Group 4, with 1.2mg/L in blood and 0.9 mg/kg in muscle tissue. Conversely, Group 2 (Chiniot diet) exhibited the lowest concentrations, at 0.4 mg/L in blood and 0.6mg/kg in muscle. The Cd bioaccumulation gradient across treatment groups followed the order: Group 4 > Group 3 > Group 2. Statistically significant differences ($P < 0.05$) were observed between the experimental groups and the control group, directly correlating with the elevated PTE levels in the sewage-irrigated diet compared to the canal water-irrigated control diet (Tab. 3.1).

The liver and kidneys were identified as the primary target organs for Cd accumulation across all treatment groups. Cadmium concentrations in the liver and kidneys were 1.76mg/kg and 0.93 mg/kg for Group 2 (Chiniot), 2.28 mg/kg and 1.37mg/kg for Group 3 (Faisalabad), and 2.99 mg/kg and 1.46 mg/kg for Group 4 (Kasur), respectively. These results indicate a significantly higher hepatic impact from the consumption of hyper-accumulative crops from Site 4 (Kasur) compared to Sites 2 and 3. While the heart exhibited the lowest overall Cd accumulation (minimum observed in Group 2 at 0.47mg/kg), other vital organs, including the brain 1.08mg/kg and lungs 1.37mg/kg, showed maximum concentration in Group 4 rats. The descending order of Cd bioaccumulation in vital organs was generally: liver > kidney > lungs > brain > heart. The difference in Cd concentration between the control and all treatment groups was statistically significant ($P < 0.05$), although no significant difference ($P < 0.05$) was found between Group 2 and Group 3 regarding hepatic accumulation.

The findings of this study corroborate the established literature identifying the kidney as a primary target organ for cadmium, prone to significant renal toxicity upon chronic exposure (Nawrot et al., 2010). However, while one study suggests that over one-third of total cadmium deposition typically occurs in the kidney (Nawrot et al., 2010). our findings demonstrate a greater hepatic accumulation of Cd compared to renal accumulation. This discrepancy suggests that dietary ingestion of Cd-contaminated sewage-irrigated crops may alter the tissue distribution profile, favoring hepatic sequestration.

Furthermore, the severity of potential health implications is directly related to exposure concentration, duration, and the chemical form of the metal. Our results confirm that the diet derived from the highly contaminated district (Kasur) induces a significantly higher Cd burden in vital organs compared to the less contaminated diet from Chiniot (Gulfam, 2021). The profound systemic impact of these metals is attributed to the promotion of oxidative stress, production of reactive oxygen species (ROS) (Navas-Acien et al., 2005; Prozialeck et al., 2006), enzyme inactivation, and the weakening of the antioxidant defense system, ultimately leading to significant weight loss (Balali-Mood et al., 2021).

**Tab. 3.1. Cd distribution and accumulation in rat's tissues and bones.**

| Tissue | Control (G1) | Chiniot (G2) | Faisalabad (G3) | Kasur (G4) |
|---|---|---|---|---|
| Liver | 0.30 ± 0.10 | 1.77 ± 0.09 | 2.28 ± 0.17 | 2.99 ± 0.21 |
| Kidneys | 0.17 ± 0.05 | 0.93 ± 0.06 | 1.37 ± 0.06 | 1.46 ± 0.05 |
| Lungs | 0.20 ± 0.05 | 0.75 ± 0.07 | 0.92 ± 0.04 | 1.38 ± 0.05 |
| Bones | 0.26 ± 0.07 | 1.22 ± 0.10 | 1.79 ± 0.13 | 2.03 ± 0.16 |
| Blood | 0.10 ± 0.02 | 0.66 ± 0.09 | 0.89 ± 0.03 | 1.28 ± 0.07 |
| Muscles | 0.13 ± 0.02 | 0.46 ± 0.08 | 0.67 ± 0.02 | 0.90 ± 0.08 |
| Brain | 0.18 ± 0.04 | 0.76 ± 0.08 | 0.92 ± 0.05 | 1.09 ± 0.14 |
| Heart | 0.14 ± 0.03 | 0.48 ± 0.07 | 0.76 ± 0.13 | 0.92 ± 0.03 |

### 3.4 Bioaccumulation of Cd in bones:

The highest concentration of Cd was observed in bones of group 4 rats was 2.03 mg $kg^{-1}$ while the lowest concentration 1.22 mg $kg^{-1}$ was observed in group 2 rats (Tab.3.1). The observed Cd concentration was recorded the highest in all treatment groups which have been provided sewage irrigated diets over the control group. The order of Cd concentration in bone of all treatment groups is given as, group4 > group 3 > group2. The difference in concentration of Cd between the bone of control group rats and treatment groups rats was significantly higher ($P<0.05$) as expected because the rats of all the treatment groups were being fed with sewage irrigated diet while the control group was receiving canal water irrigated diet.

The concentration of Cd in bones remained much higher than to some vital organs, indicating a more favorable site for Cd accumulation as it replaces Ca from bones, findings of this experiment were similar to Krishnan et al (1990), that Cd uptake in rats at higher concentrations for few weeks through drinking water tends to accumulate high concentrations of Cd in bones and kidneys without indicating any signs of damage to both bones and kidneys (Krishnan et al., 1990). Potentially toxic metals have also been associated with degenerative diseases of the bones, such as osteoporosis (Kido, 2013). In Japan, Itai-Itai disease has been associated with the accumulation of cadmium in the skeleton of the affected

individuals. Absorption of cadmium in the renal cortex was also found to interfere with enzymes involved in the regulation of specific vitamins. As a result, decreased tubular reabsorption occurred, adversely enhancing the excretion of essential elements such as phosphorus and calcium, which play basic roles in bone metabolism (Keiko & Minoru, 1991; Tang et al., 2016).

### 3.5 Distribution and bioaccumulation of Pb in rat tissues:

Owing to the higher concentration of Lead (Pb) in the sewage-irrigated diets compared to Cadmium (Cd), a correspondingly higher bioaccumulation of Pb was observed across all treatment groups. Tissue analysis revealed a distinct bioaccumulation gradient, with concentrations in blood and muscle following the order: Group 4 (Kasur) > Group 3 (Faisalabad) > Group 2 (Chiniot). Maximum Pb concentrations were recorded in Group 4, reaching 1.86mg/L in blood and 1.3 mg/kg in muscle tissue. Conversely, the lowest concentrations were observed in Group 2, at 1.03 mg/L and 0.76mg/kg, respectively. The difference in Pb concentration between the control group (canal water-irrigated diet) and all treatment groups (sewage-irrigated diet) was statistically significant ($P < 0.05$; Tab. 3.2).

Analysis of vital organs revealed that the brain was the primary target for Pb accumulation, followed by the liver and kidneys. The highest concentration was observed in the brain, with Kasur group rats (Group 4) exhibiting significantly higher levels compared to Groups 2 and 3. Pb concentrations in the brain, liver, and kidney for the Chiniot, Faisalabad, and Kasur groups were 2.19, 1.53, 1.13 mg/kg, 2.71, 1.82, 1.37 mg/kg, and 3.09, 2.66, 2.04 mg/kg, respectively. Other vital organs, including the lungs 1.44mg/kg and heart 0.96 mg/kg, also showed maximum Pb accumulation in the Kasur group. The general descending order of Pb toxicity in vital organs was: brain > liver > kidney > lungs > heart.

The high accumulation of Pb in the brain and liver confirms these tissues as critical targets for lead toxicity, directly resulting from the consumption of sewage-irrigated crops. These findings align with literature indicating that Pb, alongside other heavy metals like Cd and As, poses serious risks to hepatic function, with exposure clearly associated with liver injury (Pillai & Gupta, 2005). Furthermore, the significant retention of Pb in brain tissues corroborates studies linking lead exposure to irreversible damage to the central nervous system (CNS), including cognitive and memory dysfunction (Sankhla, Kumar, & Prasad, 2019). While literature highlights that children experience greater lead absorption and retention in brain cells compared to adults (Giedd, 2004). our study demonstrates substantial Pb accumulation in the brain tissues of adult male rats, underscoring the severe risks posed by long-term consumption of contaminated produce.

### Tab. 3.2. Pb concentration in rats consuming sewage irrigated crops

| Tissue | Control (G1) | Chiniot (G2) | Faisalabad (G3) | Kasur (G4) |
|---|---|---|---|---|
| **Brain** | 0.35 ± 0.02 | 2.19 ± 0.06 | 2.71 ± 0.04 | 3.09 ± 0.08 |
| **Liver** | 0.36 ± 0.01 | 1.53 ± 0.06 | 1.82 ± 0.03 | 2.66 ± 0.03 |
| **Bones** | 0.23 ± 0.02 | 1.49 ± 0.03 | 1.71 ± 0.02 | 2.43 ± 0.05 |
| **Kidneys** | 0.33 ± 0.04 | 1.13 ± 0.05 | 1.37 ± 0.03 | 2.04 ± 0.08 |
| **Blood (mg/L)** | 0.19 ± 0.02 | 1.03 ± 0.05 | 1.42 ± 0.03 | 1.86 ± 0.04 |
| **Lungs** | 0.18 ± 0.02 | 0.58 ± 0.03 | 0.81 ± 0.02 | 1.44 ± 0.03 |
| **Muscles** | 0.15 ± 0.01 | 0.76 ± 0.06 | 0.91 ± 0.03 | 1.30 ± 0.03 |
| **Heart** | 0.13 ± 0.03 | 0.46 ± 0.03 | 0.64 ± 0.02 | 0.96 ± 0.07 |

### 3.6 Distribution and bioaccumulation of Pb in rats' bones:

The maximum concentration of Pb was found in bones of group 4 rats was 2.43 mg $kg^{-1}$ while the lowest concentration, 1.49 mg $kg^{-1}$ was observed in group 2 rats. The observed Pb concentration was recorded highest in all treatment groups which have been provided sewage irrigated diets over the control group. The order of Pb concentration in bone of all treatment groups is given as, group4 > group 3 > group2. The difference in concentration of Pb between the bone of control group rats and treatment groups rats was significantly higher ($P<0.05$) as expected because the rats of all the treatment groups were being fed with sewage irrigated diet while the control group is receiving canal water irrigated diet.

Potentially toxic metals particularly Pb and Cd have also been associated with degenerative diseases of the bones such as osteoporosis (Kido, 2013). The rats have been exposed to a Pb contaminated diet sub chronically in a study, and showed high concentration of Pb in their bones, which is similar with Maldonado-Vega et al (1996), as the chronically exposed rats with Pb exhibit much higher concentration of Pb in their bones. Similarly another group of lactating rats on exposure with Pb showed a maximum concentration of Pb in their bones (Maldonado-Vega, Cerbón-Solorzano, Albores-Medina, Hernández-Luna, & Calderón-Salinas, 1996).

This self-explanatory visualization maps the toxicological gradient in Wistar rats, highlighting the liver as the primary sink for extreme bioaccumulation and physiological atrophy. While the renal and pulmonary systems exhibit high to moderate susceptibility, the minimal mass loss was observed in the brain. The color-coded matrix (Red: Extreme to Yellow: Minimal) effectively integrates Bioaccumulation Index (BAI), metabolic stress, and gravimetric data to provide a comprehensive biological endpoint profile (Fig. 3.3).

**Tab. 3.3. Bioaccumulation index of Cd in different body parts of rats.**

| Body parts | BAI-Chiniot G2 | BAI-Faisalabad G3 | BAI-Kasur G4 |
|---|---|---|---|
| Muscles | 2.67 | 4.31 | 6.10 |
| blood | 5.35 | 7.61 | 11.35 |
| Liver | 4.89 | 6.60 | 8.98 |
| Kidneys | 4.58 | 7.24 | 7.76 |
| Lungs | 2.83 | 3.69 | 6.00 |
| Heart | 2.33 | 4.33 | 5.40 |
| Brain | 3.24 | 4.11 | 5.04 |
| Bones | 3.71 | 5.88 | 6.81 |

### 3.7 Bioaccumulation index (BAI) of Cd and Pb

The Bioaccumulation Index (BAI) analysis demonstrated that rats consuming sewage-irrigated crops from Site 4 (Kasur) exhibited the highest affinity for Cadmium (Cd) and Lead (Pb) accumulation across all monitored tissues compared to Groups 2 and 3.

#### 3.7.1 Cadmium (Cd) Accumulation

Group 4 rats displayed the highest BAI for Cd in blood (11.35) and muscle (6.1), while Group 2 (Chiniot) showed the lowest affinity (5.4 and 2.67, respectively). Among vital organs, the liver consistently exhibited the highest BAI for Cd across all groups, peaking in Group 4 (8.98). The order of Cd BAI varied by group: Group 2: Liver > Kidney > Brain > Lungs > Heart, Group 3: Kidney > Liver > Heart > Brain > Lungs, Group 4: Liver > Kidney > Lungs > Heart > Brain (Tab. 3.3).

#### 3.7.2 Lead (Pb) Accumulation

For Lead, the brain demonstrated the highest susceptibility to bioaccumulation, reaching a maximum BAI in Group 4 (7.92). In contrast, the heart in Group 2 showed the minimum Pb affinity. The descending order of Pb BAI in vital organs generally followed: Brain > Liver > Heart > Lungs > Kidney, with the exception of the lungs in Group 4, which ranked directly below the brain in accumulation capacity. Notably, the highest overall BAI for Pb was found in the bones of Group 4 rats (9.71) (Tab. 3.4), indicating significant skeletal deposition and potential structural compromise.

The superior bioaccumulation of Cd and Pb in Group 4 (Kasur) rats compared to Groups 2 and 3 confirms a direct dose-response relationship governed by the contamination gradient. However, the specific BAI values differ from studies conducted in contrasting environmental conditions. For instance, our observed hepatic Cd BAI is however, higher than reported by Peera at al. (2023), in a similar study in China, likely due to higher bioavailability of metals in the alkaline, low-organic-matter soils typical of

Punjab, which facilitates greater uptake despite lower total soil concentrations. (Peera, Agraharam, & Jamakala, 2023).

The high BAI values recorded in this study are alarming. Tóth et al. (2016), in a review of EU soils, highlighted that even minor contamination triggers significant bioaccumulation in leafy vegetables. Our findings demonstrate that in Faisalabad and Kasur, this accumulation is multiplied within the food chain(Tóth, Hermann, Da Silva, & Montanarella, 2016). The severe reduction in organ weights (liver and kidney) observed in Group 4 rats is consistent with the pathological impacts described by Prozialeck et al. (2006) and Navas-Acien et al. (2005), who demonstrated that reactive oxygen species (ROS) generation by Pb and Cd disrupts cellular integrity. Furthermore, the magnitude of weight loss matches observations by Balali-Mood et al. (2021) on chronic systemic toxicity, but exceeds those reported by Muktar et al. (2021), likely due to the specific composition of the diet combining both contaminated spinach and cabbage, which synergistically increased the toxic load (Muktar et al., 2021). The exposure concentration, time and chemical form of a metal present in the food are directly related to the severity of potential health implications to the individual(Navas-Acien et al., 2005; Prozialeck et al., 2006).

**Tab. 3.4. Bioaccumulation index of Pb in different body parts of rats.**

| Body parts | BAI-Chiniot G2 | BAI-Faisalabad G3 | BAI-Kasur G4 |
|---|---|---|---|
| Muscles | 4.09 | 5.04 | 7.69 |
| blood(mg/L) | 4.33 | 6.33 | 8.60 |
| Liver | 3.20 | 4.00 | 6.32 |
| Kidneys | 2.41 | 3.14 | 5.19 |
| Lungs | 2.26 | 3.58 | 7.15 |
| Heart | 2.66 | 4.08 | 6.58 |
| Brain | 5.33 | 6.83 | 7.92 |
| Bones | 5.56 | 6.56 | 9.71 |

**Tab. 3.5. Hematological parameters of rats consuming sewage irrigated crops.**

| Parameter | Units | Control (G1) | Chiniot (G2) | Faisalabad (G3) | Kasur (G4) |
|---|---|---|---|---|---|
| HGB | g/dl | 15.00±0.37$^{a}$ | 12.80±0.40$^{b}$ | 10.41±0.02$^{c}$ | 9.34±0.16$^{d}$ |
| RBC | ×106/µL | 9.18±0.37$^{a}$ | 7.23±0.12$^{b}$ | 6.00±0.21$^{c}$ | 5.41±0.04$^{d}$ |
| WBC | ×103/µL | 7.87±0.14$^{d}$ | 9.53±0.05$^{c}$ | 11.25±0.05$^{b}$ | 14.33±0.10$^{a}$ |
| PLT | ×103/µL | 913.67±5.55$^{a}$ | 793.00±4.93$^{b}$ | 716.67±3.84$^{c}$ | 618.00±2.65$^{d}$ |
| HCT | % | 49.42±0.56$^{a}$ | 42.33±0.88$^{b}$ | 38.00±0.58$^{c}$ | 34.00±0.58$^{d}$ |
| MCV | fL | 58.33±0.88$^{b}$ | 61.33±0.33$^{a}$ | 62.00±0.58$^{a}$ | 62.33±0.33$^{a}$ |
| MCH | pg | 17.98±0.11$^{b}$ | 18.20±0.03$^{a}$ | 18.36±0.07$^{a}$ | 18.57±0.03$^{a}$ |
| MCHC | g/dl | 32.03±0.37$^{a}$ | 30.20±0.06$^{b}$ | 27.00±0.58$^{c}$ | 25.33±0.88$^{d}$ |

### 3.8 Impact on Hematological Profiles

Sub-chronic exposure to PTE-contaminated diets induced significant alterations in the hematological parameters of all treatment groups ($P \leq 0.05$). A marked dose-dependent decrease was observed in Red Blood Cell (RBC) counts, Hemoglobin (HGB) levels, Hematocrit (HCT %), and Platelet (PLT) counts compared to the control group. The most severe decline was recorded in the Kasur group, where HGB and RBC levels fell significantly, indicating a state of progressive anemia. Conversely, White Blood Cell (WBC) counts showed a significant increasing trend across all treatment groups, with the highest values observed in rats fed the Kasur district diet (Tab. 3.5). These fluctuations in blood indices were directly proportional to the concentration of Cd and Pb in the crops, confirming that sewage-irrigated produce exerts a strong hematotoxic effect.

Cadmium and lead toxicity can lead to a 20% reduction in erythrocytes and a 30% reduction in hemoglobin (Nikolić et al., 2015). In current study, the similar pattern of reduction of both RBCs and HGB was followed as stated by Nikolic et al (2015). This simultaneous decrease in RBC, HGB, and HCT indicates that dietary Cd and Pb interfere with the erythropoietic system. Lead (Pb) is a well-known inhibitor of $\delta$-aminolevulinic acid dehydratase (ALAD), an enzyme essential for heme synthesis. Its inhibition leads to reduced hemoglobin production and the shortening of the RBC lifespan (Abadin, 2005). Furthermore, Cadmium (Cd) can trigger hemolysis and interfere with iron absorption in the gastrointestinal tract, compounding the anemic effect. Another study also showed that even acute lead intoxication can significantly decrease blood hematocrit (Hct), red blood cells (RBCs) and hemoglobin (Hb) (Bersenyi, Fekete, Szőcs, & Berta, 2003; Terayama, 1993) Lead in the blood binds to hemoglobin rather than the red blood cell membrane (Abadin, Ashizawa, Llados, & Stevens, 2007). Cd exposure also enhances the TLC (Total Leucocytes Count). This significant increase TLC serves as a diagnostic marker of an ongoing inflammatory response and immune system activation. This "immunological shift" is likely a defense mechanism against the oxidative stress and tissue damage caused by metal accumulation in

the liver and kidneys. The reduction in PLT (thrombocytopenia) further suggests that sub-chronic exposure to these metals may cause bone marrow suppression, hindering the production of essential clotting cells (Ellis, 1981). These findings align with the systemic stress observed in the organ weight data, providing a holistic view of the PTE-induced physiological decline. A further effect of cadmium on blood cells is hemolysis leading to anemia, insufficient erythropoietin and iron deficiency (Horiguchi, Oguma, & Kayama, 2011).

Our findings contribute to scientific knowledge by validating that untreated sewage irrigation causes mixed-metal toxicity that overrides normal physiological compensatory mechanisms in the hematological system. Practically, this highlights the need for blood monitoring of farmers and consumers in high-risk zones like Kasur.

**Tab. 3.6. Biochemical Parameters of rats consuming sewage irrigated crops.**

| Parameter | Units | Control (G1) | Chiniot (G2) | Faisalabad (G3) | Kasur (G4) |
|---|---|---|---|---|---|
| ALT | U/L | $35.00\pm1.73^{d}$ | $49.00\pm1.73^{c}$ | $54.33\pm1.45^{b}$ | $65.67\pm1.76^{a}$ |
| AST | U/L | $90.00\pm4.93^{d}$ | $152.00\pm5.51^{c}$ | $178.00\pm2.65^{b}$ | $205.00\pm3.06^{a}$ |
| ALP | U/L | $51.67\pm1.76^{d}$ | $66.67\pm2.60^{c}$ | $76.33\pm0.88^{b}$ | $92.00\pm2.08^{a}$ |
| TP | mg/dl | $6.37\pm0.09^{a}$ | $5.09\pm0.12^{b}$ | $4.35\pm0.04^{c}$ | $3.40\pm0.02^{d}$ |
| ALB | mg/dl | $3.77\pm0.09^{a}$ | $2.82\pm0.05^{b}$ | $2.32\pm0.06^{c}$ | $1.77\pm0.03^{d}$ |
| CRE | mg/L | $9.04\pm0.26^{d}$ | $11.17\pm0.02^{c}$ | $11.73\pm0.06^{b}$ | $13.50\pm0.10^{a}$ |

### 3.9 Biochemical assay:

The serum biochemical parameters alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), creatinine (CRE), total serum protein (TP), and albumin (ALB) exhibited significant variations ($P < 0.05$) directly proportional to the heavy metal load in the diet. The increasing trend in ALT, AST, ALP, and CRE, paired with a decrease in TP and ALB, confirms that chronic consumption of sewage-irrigated crops induces severe functional impairment of the liver and kidneys. These impacts were getting worse from group 2 to group 4 rats, as the group 2 was exposed with comparatively less contaminated crops regarding group 4 (Tab. 3.6).

The observed increase in ALT and AST are consistent with the findings of Kim et al. (2021), who reported that Cd and Pb exposure causes severe disruption in hepatic parenchyma, leading to the leakage of these enzymes into the bloodstream, a conclusive marker of liver injury(Kim, Ock, Moon, & Park, 2021). Furthermore, our results align with Bersenyi et al. (2003), who identified that Cd primarily elevates AST, while Pb contributes significantly to ALT increase, reflecting differential pathological impacts on liver parenchyma.(Bersenyi et al., 2003). These findings are supported by Pillai and Gupta (2005), who

established a clear association between Cd/Pb exposure and hepatic dysfunction (Pillai & Gupta, 2005). The significant rise in serum creatinine (CRE) in Group 4 rats confirms disruption in kidney filtration capacity, corroborating Prozialeck et al. (2006), who reported that Cd-induced proximal tubule damage leads to diminished glomerular filtration rates. Conversely, the decrease in TP and ALB indicates impaired protein synthesis due to hepatocyte damage and excessive protein loss through damaged kidneys, a pattern consistent with Muktar et al. (2021).

This study advances scientific knowledge by quantitatively mapping the development of biochemical damage from low-contaminated (Chiniot) to highly-contaminated (Kasur) zones, providing a robust model for risk assessment. However, a regular serum biochemical screening should be obligatory for individuals in industrial agriculture to detect organ damage before it becomes irreversible.

## Conclusion

The study was notably distinctive in its approach, utilizing an animal model to directly consume sewage-irrigated food under sub-chronic exposure conditions, rather than relying solely on conventional health risk assessment models. This approach bridges the gap between environmental monitoring and clinical toxicity, offering a realistic assessment of the biological 'tipping point' in the food-chain-to-consumer pathway. The findings revealed significant adverse effects, including reductions in body weight and alterations in hematological and biochemical parameters. Elevated concentrations of cadmium (Cd) and lead (Pb) were detected in the treated groups, with the highest levels observed in rats fed the Kasur group diet. Cd accumulation was particularly pronounced in the liver and kidneys, corroborated by abnormal liver function tests and elevated blood creatinine levels, indicating hepatic and renal damage.

Furthermore, the brain appeared especially susceptible to Pb toxicity, as its concentration was markedly higher in the brain tissues of all treated rats. These results underscore the need for further investigation into potential neurological disorders arising from chronic consumption of sewage-irrigated crops. Bone tissue was also adversely affected, with significantly increased levels of Cd and Pb, suggesting that these metals may displace calcium (Ca) and contribute to bone weakening in exposed individuals. To validate these findings, it is recommended to assess blood calcium levels in future studies.

In light of these observations, the implementation of sustainable metal remediation strategies in conjunction with sewage irrigation is strongly advised to mitigate the associated health risks and ensure safer cultivation of food crops.

## Declarations

# Competing Interest

The author has no competing interest.

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# Figures

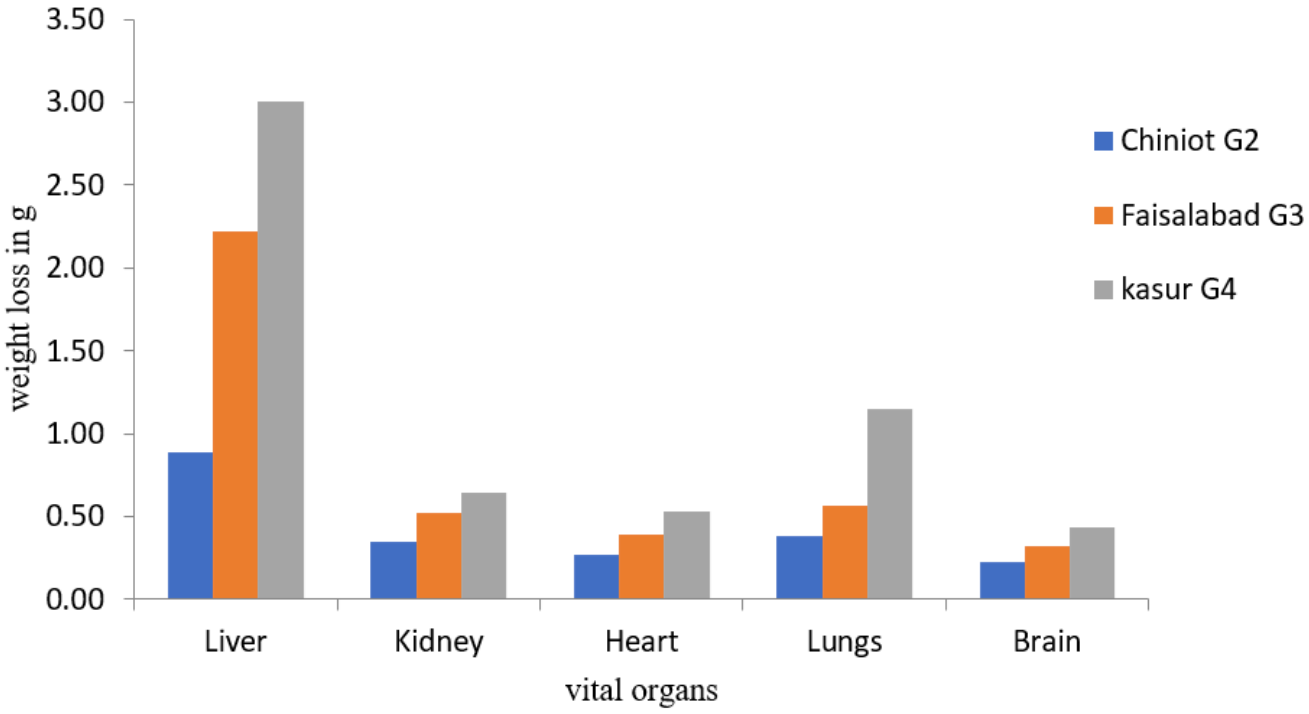



**Figure 1**

**Fig. 3.1. Weight loss (over the control G1) in vital organ of rats consuming sewage irrigated crops.**

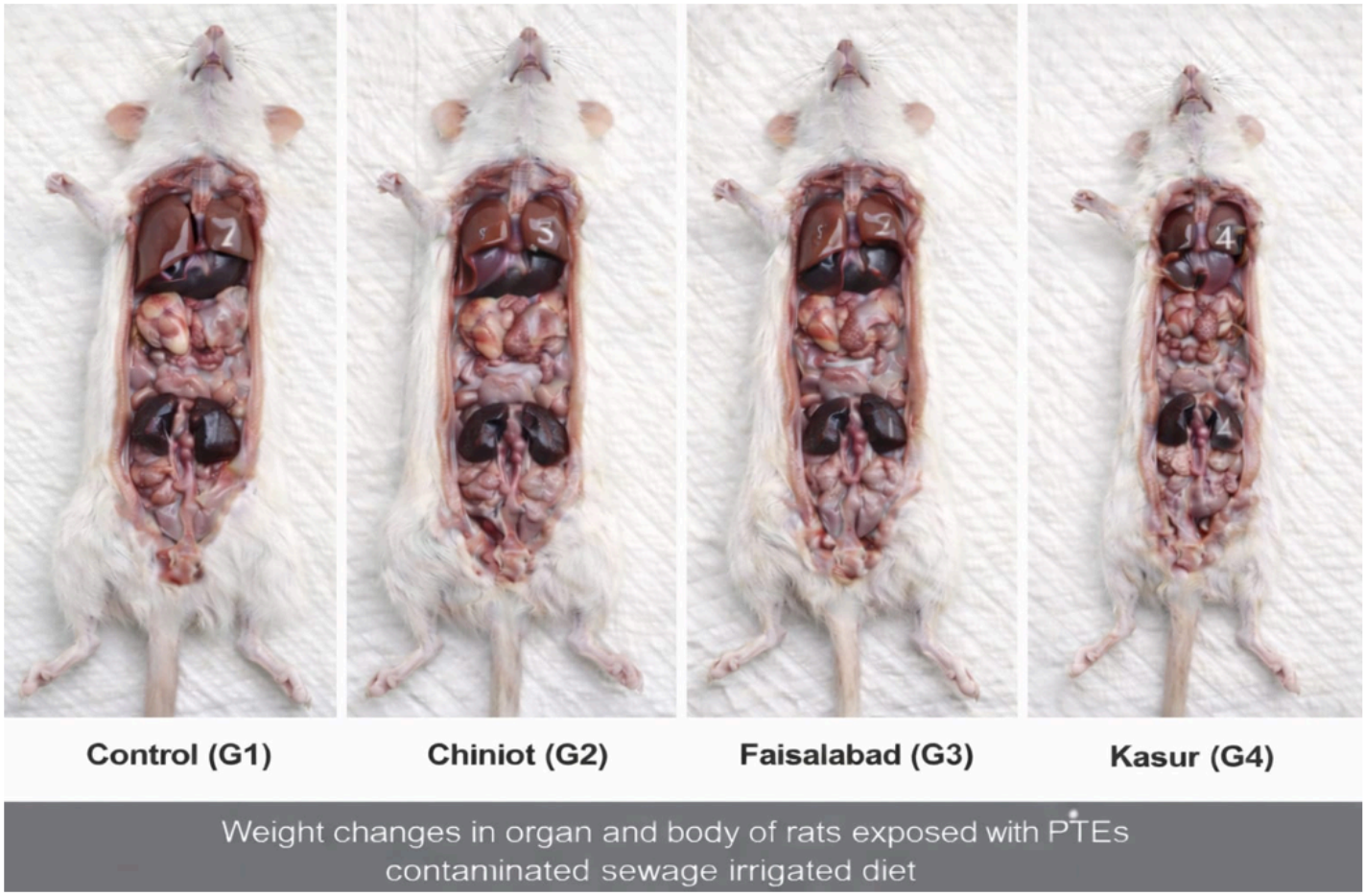



Figure 2

**Fig. 3.2. Impacts of sewage irrigated crops on organs and body weights of Wistar rats.**

| Organ | Bioaccumulation Index (BAI) | Weight Loss Severity | Metabolic Stress | Overall Toxicity |
|---|---|---|---|---|
| Liver |  |  |  | EXTREME |
| Kidneys |  |  |  | HIGH |
| Lungs |  |  |  | MODERATE |
| Heart |  |  |  | LOW |
| Brain |  |  |  | MINIMAL |

Figure 3

Fig. 3.3. Heatmap of Organ Specific Susceptibility and Toxicological Gradient in Rats.