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Histological based sub-chronic toxicity testing of target heavy metals of crude oil spill; Using the histo-morphometry of liver of Wistar rat

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Abstract

This study was aimed to evaluate in histological term, the suc-chronic toxicity of some target crude oil contaminants on the histo-morphometry of the liver of exposed Wistar rats. The following target chemicals (TCs; cadmium (Cd), chromium (Cr), copper (Cu), lead (Pb) and nickel (Ni) were selected based on findings from literature review of chemical analysis done on crude oil spill site. Thirty (30) inbred male Wistar rats of average weight 150-200g (5 for control and 25 for experimental – 5 for each of the five TC treated groups). Rats were later sacrificed and the target organ (liver) excised and used for qualitative histological evaluation. Gross anatomical assessment showed that there was no significant difference (P> 0.05) when correlating weight gain between the treated and control groups. Histological evaluation showed the following major lesions: glomerular congestion, degeneration and necrosis; tubular degeneration and necrosis; Interstitial inflammation, hemorrhage and necrosis. This study gives credence to the fact that histology-based evidence is a veritable tool for assessing sublethal level of environmental stressors in the certification of toxicity.

Keywords: Wistar Rat; Liver; Histology; Sub-Chronic Toxicity; Toxicity; Heavy Metals

1. Introduction

Toxicology is a branch of biology, chemistry, and medicine (more specifically pharmacology) concerned with the study of the adverse effects of chemicals on living organism (Schrager, 2006). It also studies the harmful effects of chemical, biological and physical agents in biological system that establishes the extent of damage in living organisms. The relationship between dose and its effects on the exposed organism is of high significance in toxicology. Factors that influence chemical toxicity includes; the dosage (and whether it is acute or chronic), the route of exposure, the species, age, sex and environment.

Toxicity tests can measure lethal and/or sublethal effects. These effects are known as measurement endpoints: that is, they are ecological attributes that may be adversely affected by exposure to site contaminants that are readily measurable. In addition, each measurement endpoint is closely related to an assessment endpoint. Because of this close relationship, a measurement endpoint can approximate or represent the assessment endpoint if the assessment endpoint is not amenable to direct measurement (USEPA, 1992). Based on the measured end points, toxicity testing can be divided into acute toxicity testing, sub-chronic toxicity testing and chronic toxicity testing. Sub-chronic toxicity testing was applied for this study, which is defined as a prolonged toxicity test for 14 day. This prolonged toxicity test may be used in place of the acute toxicity test if a longer observation period is considered appropriate (OECD, 1984), for example if testing highly lipophilic, poorly water-soluble substances, and/or the reporting of additional information

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is considered necessary. The principle of the test is that threshold levels of lethal and other observed effects and NOEC are determined at intervals during the test period (OECD, 1992a).

Organism exposure to hazardous chemicals causes increase in the levels of stress proteins, which induces specific detoxification system responses, reflecting their compensatory potential. When these systems are overwhelmed, it causes sublethal effect which usually begins as biochemical alterations at the molecular levels of biological organization. This change might not impair cellular function until when it results in the formation of a lesion, which is any structural damage or alterations in an organ, tissue or cell of an organism due to an injurious stimulus. Lesion diagnosis is a report on the qualitative and/or quantitative gross morphological alterations of organs or histo-morphological alteration of tissues, cells and organelles, including the histo-chemical changes that occur at the molecular level of biological organization. Diagnostic statement of a lesion is based on the predominant lesion(s) in the tissue, while lesion description can be based on the severity (mild moderate or severe), distribution (focal, multi-focal or diffuse), location (intracellular, epithelial or interstitial) and pathogenesis (adaptive degenerative, inflammatory, and neoplastic). In so far that sublethal lesions of toxic substances is insidious and usually begins at the subcellular levels of biological organization, even before its manifestation of impairment of the physiology of the affected cell, histology as an assessment tool is therefore a gold standard in certification of toxicity – in a laboratory toxicological setting or pollution in an ecotoxicological setting. It is noteworthy that physiologic and biochemical changes are predominantly due to structural abnormality of tissue which histopathology investigates. Hence, lesion diagnosis is a gold standard in diagnosis of disease pathogenesis and pathognomonic histopathologic features. Some lesions are pathognomonic histopathological features that are specifically distinctive or characteristic of some diseases or pathological conditions.

The liver is unique in that it receives blood from two sources: the *hepatic artery* and the *portal vein*. As these vessels enter the liver, their terminal branches run alongside branches of the bile ducts and course together throughout the liver parenchyma within *periportal area*, which consists of four vessels: *hepatic artery*, *portal vein*, *bile ductile* and *lymphatic vessel*, forming the *portal area*. The periportal blood vessels supply's the liver parenchyma by flowing through the liver *sinusoids* to the central vein, which lies in in the centre of the *centrilobular area*.

The blood supply to the liver parenchyma flows from the portal area to the central veins. Accordingly, the hepatic parenchyma of classical liver lobule is divided into 3 zones (Allison et al., 2022):

- **Zone 1** or the periportal (peripheral) area is closest to the arterial and portal blood supply and hence bears the brunt of all forms of toxic injury.
- **Zone 2** is the intermediate midzonal area.
- **Zone 3** or the centrilobular area surrounds the central vein and is most remote from the blood supply and thus suffers from the effects of hypoxic injury.

The sinusoids are flanked by hepatocytes. Hepatocytes are the chief functional cells of the liver and perform an astonishing number of *metabolic*, *endocrine* and *secretory functions*. Roughly 80% of the mass of the liver is contributed by hepatocytes. In three dimensions*, hepatocytes are arranged in plates that anastomose with one another*. The cells are polygonal in shape and their sides can be in contact either with sinusoids (*sinusoidal face*) or neighbouring hepatocytes (*lateral faces*). A portion of the lateral faces of hepatocytes is modified to form *bile canaliculi. Microvilli are present abundantly on the sinusoidal face* and project sparsely into bile canaliculi (Allison et al., 2022).

The liver is a digestive gland and a key organ which controls many life functions. It plays a prominent role in mammalian physiology, both in anabolism (proteins, lipids and carbohydrates), catabolism (nitrogen, glycogenolysis, detoxification) and it serves as a storage site for many substances mainly glycogen (Hinton and Lauren, 1990; Takashima and Hibiya 1995; Akiyoshi and Inoue, 2004). Contaminants, such as heavy metals, pesticides, hydrocarbons and physico-chemical water parameters can alter the structure and metabolism of the liver (Brusle et al., 1996). Histological alterations common in liver following exposures to toxicants include cloudy swelling, atrophy, hepatocyte hypertrophy, necrosis, hyperplasia, vacoular degeneration, fatty degeneration, congestion and bile stagnation (Takashima and Hibiya, 1995).

The health effects of toxic substances and hazardous wastes are not yet fully understood. Research to better understand how these exposures may impact health is ongoing. Meanwhile, efforts to reduce exposures continue. Reducing exposure to toxic substances and hazardous wastes is fundamental to environmental health.

2. Materials and methods

The study was a sub-chronicity testing for 14 days. Thirty (30) inbred male Wistar rats were obtained from the animal house of the Department of Anatomy, Faculty of Basic Medical Sciences, University of Benin for this study. The rats were divided into experimental and control groups:

- Control Group: Five (5) rats were used for this group. They were untreated with target chemical (TC), and were only given only water and food.
- Experimental Group: Twenty-five (25) rats were used for the experiment group. They were treated with the study TCs. This group was sub-divided into 5 Wistar rat per TC treated group of Cd, Cr, Cu, Ni and Pb.

In estimation of the rat exposure dose (RED) for the study, OEDC (2001) reference oral LD_{50} dose for Wistar Rat (in mg/Kg of body weight) of the TCs – Cd (63mg/kg); Cr (46 mg/kg), Cu (481 mg/kg); Nickel (300 mg/kg) and Pb (600 mg/kg), was used as a guideline standard for the upper limits of dose administration (OECD, 2001). Ten times (10x) the TC concentration that is above maximum allowable toxicant concentration (MATC) for surface fresh water, but below the median lethal dose (LD_{50}) reference concentration for Wistar Rats was used as the guideline for estimation of the RED (Hounkpatin et al., 2013; Thinkratok, et al., 2014). Thus analytical grade metallic salts of the TCs concentrations that are 10x >MATC standard per TC, but below the LD50 per TC for Wistar Rat was dissolved in 100litres of distilled water to make the stock solution. 1ml of the stock, for each of TCs, was administered orally/day for 14 days to the test rats (Thinkratok, et al., 2014). Therefore, RED in mg/ml for this study was: Cd (0.0001 mg/ml), Cr(0.01 mg/ml), Cu(0.02 mg/ml), Ni(0.25 mg/ml) and Pb(10.0 mg/ml). Oral route was chosen as the route of administration of the test solution because it is the most common mode of exposure of the target toxicants (ATSDR, 2004; ATSDR, 2007; ATSDR, 2012a, 2012b).

All animals used in this study were handled with regards to international, natural and institutional guidelines for care and use of laboratory animals in biomedical research as promulgated by the Canadian Council of Animal Care (CCAC, 1984). Study animals were housed in cages with wire bar lids used to hold water bottle and feeds to prevent contamination with urine or feces. Bedding was placed directly into the shoe box cage to allow the absorption of urine. Test animals were kept in well-ventilated room at ambient temperature of 28.0±2.0 ^{o}C under 12hour light/dark cycle well fed with food and water ad libitum. Generally, the study was conducted in accordance with the recommendations from the declaration of Helsinki on guiding principles in care and use of animals (Obianime and Roberts, 2009).

Histological tissue processing and qualitative analysis of prepared tissue slides was done at the Histology Laboratory of the Department of Anatomy, School of Basic Medical Sciences, University of Benin. Resected target organ of liver was collected in vials filled with preservative (10% neutrally-buffered formalin solution), and transported to the University of Benin Histology laboratory for tissue processing and staining. The prepared tissue slides were used for quality histological evaluations. (Drury and Wallington, 1980; Allison and Paul, 2014; Allison and Paul, 2018)

3. Results

3.1. Gross Anatomical Assessment

The Wistar rat sub-chronic toxicity showed that, rats exposed to the daily rat exposure doses (RED) of Cu1, Ni1 and Pb1 died. Only those exposed to the daily doses of Cd1, Cr1 and control survived the 14day experiment. Table 1 showed that there was no significant difference ($P \geq 0.05$) when correlating weight gain between the treated and control groups.

Table 1 correlating weight gain between experimental and control groups using t test analysis

Group	No of Male Rats	Daily RED (mg/ml)	Exp. Days	Mean Wo (g)	mean W_1 (g)	Weight Gain	Sig. (2-tailed)	
Control		Nil	14	175.5	208.4	32.9	0.300	Not Significant
Cd1	5	0.0001	14	180.6	212.3	31.7	0.419	Not Significant
Cr1	5	0.01	14	175.3	203.6	28.3	0.215	Not Significant
Cu1	5	0.02	14	198.4	228.4	30.0	0.216	Not Significant
Ni1	5	0.025	14	201.0	230.4	29.4	0.650	Not Significant
Pb1	5	0.01	14	180.7	210.5	29.8	0.188	Not Significant

Key: TC = RED – Rat Exposure Dose; Exp. = Experiment; W0 = Initial Weight; W1 = Final Weight; Cd1, Cr1, Cu1, Pb1 and Ni1 = Heavy metal exposure concentration; Conc = Concentration; Sig: Significance; Mean values ($p<0.05$) are significantly different

3.2. Histology

Plate A-D are observed micrographs of Liver of Wistar rats:

3.2.1. ***Plate A****: A micrograph of the control Wistar rat*

- **Diagnostic Lesion**: Normal Architecture
- **Diagnostic Note**: The liver micrograph shows the centrilobular area with normal histological architecture, which consists of organized lobule with a central vein, hepatocytes arranged in cords and capillary sinusoids running between the rows of cord

3.2.2. ***Plate B****: A micrograph of Wistar rat exposed to Pb*

- **Diagnostic Lesion**: Hepatocyte hypertrophy with associated sinusoidal dilation
- **Diagnostic Note**: The liver micrograph shows the periportal area, which consist of diffused hypertrophied hepatocytes with mild sinusoidal dilation. The hepatocytes'cytoplasm is enlarged and pale with a ground glass appearance. It is associated with sinusoidal dilation – the sinusoidal spaces are enlarged when compared with the control micrograph. Other observed histological alterations include:
    - **Portal vein epithelial disruption**: The epithelium of the portal vein is disrupted resulting in extravasation of blood into the interstitium.
    - **Coagulative necrosis**: It is characterized by areas of loss of hepatocytes, with observed karyolysis and replacement with erythrocytes

3.2.3. ***Plate C****: A micrograph of the Wistar rat exposed to Cd*

- **Diagnostic Lesion**: Mild hepatocytes hydropic degeneration with associated coagulative necrosis and hepatocyte hypertrophy
- **Diagnostic Note**: The liver micrograph shows the periportal area, which consist of mild hydropic degeneration
- hepatocytes were swelling and rounding up with watery oedematous wispy rarified cytoplasm that is less eosinophilic than the normal cells. Some of the injured hepatocytes are undergoing severe form of hydropic degeneration (i.e. ballooning degeneration) which is characterized by their cytoplasm appearing swollen and granular, which tends to condense around the nucleus. Other observed histological alterations include:
    - **Coagulative necrosis**: It is characterized by areas of loss of hepatocytes, with observed karyolysis and replacement with erythrocytes
    - **Hepatocyte hypertrophy**: The affected hepatocytes cytoplasm have a pale, ground glass appearance with associated karyomegaly and multinucleated hepatocytes.
    - **Dropout ballooning necrosis:** A small cluster of degenerating hepatocytes show dropout ballooning necrosis, which characterized by kariolysis of their nucleus.

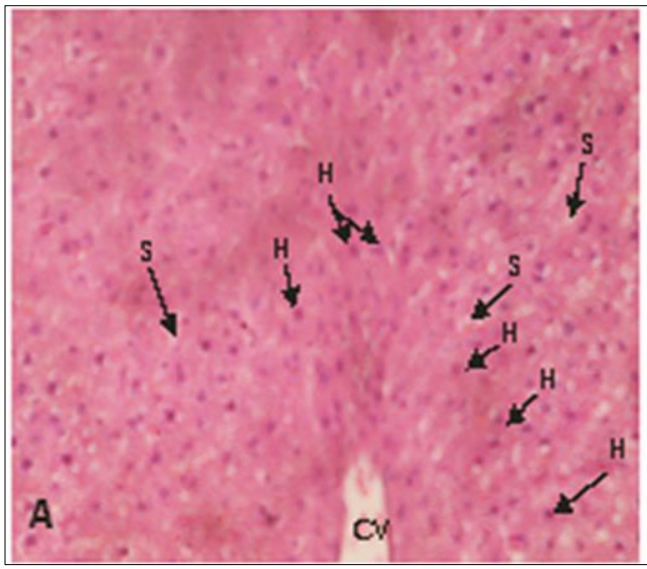

Figure 1 Photomicrograph (H and E) 400x magnification of normal architecture of the liver of the control wistar rat showing hepatocytes (H) Sinusoids (S) central vein (CV) and bile duct (BD)

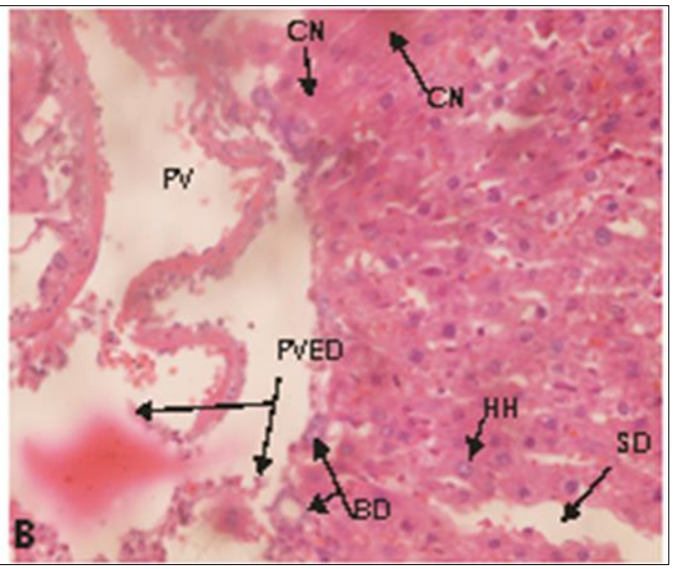

Figure 2 Photomicrograph (H and E) 400x magnification of liver exposed to pb showing the periportal area which consist of portal vein (PV) bile ductile, portal vein epithelial disruption (PVED) coagulative necrosis (CN) hypertrophied hepatocytes (hh) and sinusoid dilation (SD)

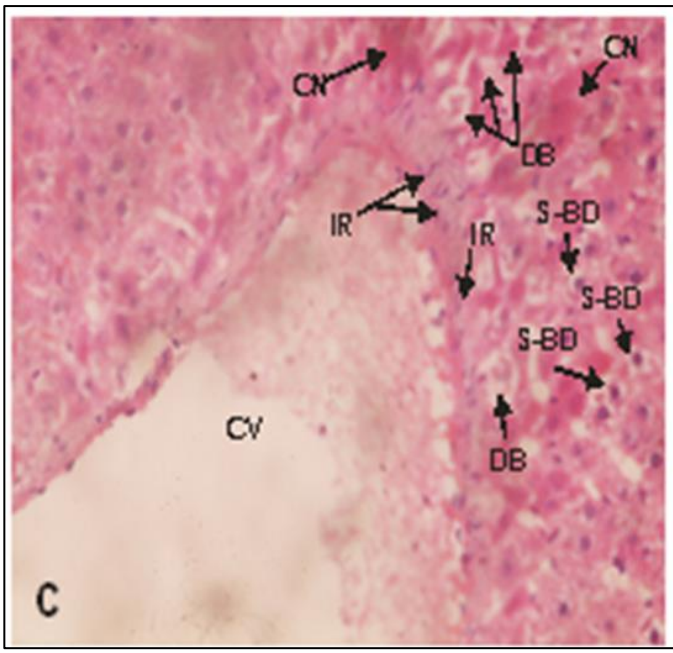

Figure 3 Photomicrograph (H and E) 400x magnification of liver exposed to Cd showing the Centrilobular area which consist of central vein (CV) coagulative necrosis (CN) hypertrophied hepatocytes (IR) dropout ballooning (DB) and swelling of the hepatocyte due to ballooning Degeneration (S-BD)

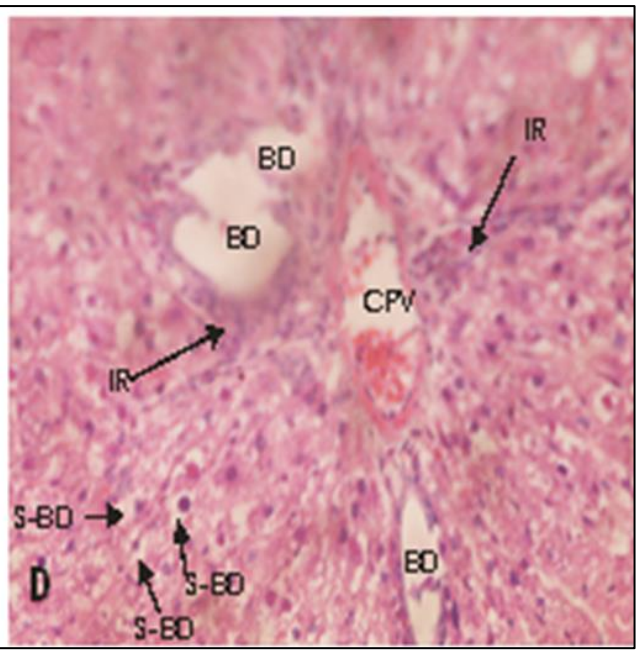

Figure 4 Photomicrograph (H and E) 400x magnification of liver exposed to cr showing the periportal area which consist of the congested hepatic vein (CHP) bile ductules (BD) cell infiltration (IR) swelling hepatocytes with ballooning degeneration (s-DB)

3.2.4. ***Plate D****: A micrograph of the Wistar rat exposed to Cr*

- **Diagnostic Lesion**: Moderate Hepatocytes hydropic degeneration with associated inflammation
- **Diagnostic Note**: The liver micrograph shows the periportal area, which consist of moderate hydropic degeneration – hepatocytes were swelling and rounding up with watery oedematous wispy rarified cytoplasm

that is less eosinophilic than the normal cells. Some of the injured hepatocytes are undergoing severe form of hydropic degeneration (i.e. ballooning degeneration) which is characterized by their cytoplasm appearing swollen and granular, which tends to condense around the nucleus. Other observed histological alteration include:

Inflammatory cells infiltration: There is presence of multiple foci of inflammatory cells.

4. Discussion

4.1. Gross Assessment

This involves the weight relationship between the control and experimental group. There was no significant difference in mean weight gain between experimental Wistar Rats groups exposed to TCs Rat Exposure Doses (REDs) of Cd1, Cr1, Cu1, Ni1 and Pb1, and the control group. This implies that the RED does not have significant effect on the weight of Wistar rats, which is consistent with other studies in which Wistar Rats were exposed to heavy metals. (Melo et al., 1998; Honglin et al., 2017).

4.2. Histology

The liver is the primary target organ following acute or chronic toxicity of exposure. The uptake of heavy metals into the liver is critical for the development of overall toxicity induced by heavy metals. Approximately half of heavy metals absorbed systemically are rapidly accumulated in the liver, which resulted in the reduced availability of heavy metals to other organs like the kidneys, lungs and testes, which are more sensitive to its toxic actions (DelRaso et al., 2003).

In this study, the liver showed variable histological alterations in the degree of liver cell injury but it was most marked in centrilobular area (zone 3 of classical lobule) with mildly injured hepatocytes, which appear swollen with granular cytoplasm that tends to condense around the nucleus (S-BD - ballooning degeneration). Observed diagnostic lesions include:

4.2.1. Hepatocyte hypertrophy

Hepatocyte hypertrophy is a form of cytologic alteration that is diagnosed based on an observable increased in size of hepatocytes compared with concurrent control liver. It is most readily apparent when it has the commonly occurring centrilobular distribution pattern; when it is panlobular, comparison with concurrent controls can provide diagnostic confirmation (NTP, 2014; Allison et al., 2022). When mild, detection of hepatocyte hypertrophy may be difficult, but its identification is facilitated at low magnification. It most frequently affects centrilobular hepatocytes, depending upon the xenobiotic and the dose administered, although the hypertrophy can extend into the middle of the hepatic lobule or become panlobular. It is also possible for periportal hepatocytes to be primarily affected, though care must be taken not to confuse periportal hypertrophy with processes that result in shrinkage of centrilobular hepatocytes (e.g., glycogen depletion) (NTP, 2014; Allison et al., 2022). In this study, hypertrophic hepatocyte cytoplasm was pale with ground glass appearance and associated with karyomegaly and multinucleated hepatocytes. This was consistent with chronic exposure of Wistar rat liver to some non-genotoxic hepatic toxicants. (NTP, 2014).

4.2.2. Hepatocytes hydropic degeneration

Hydropic degeneration of hepatocytes describes the swelling and rounding up of injured hepatocyte which is considered as a sign of progression to cell death. The injured cells present a clear field in cytoplasm which are spaces for accumulating massive glycogen (NTP, 2014; Allison et al., 2022). Granules are formed around the nuclei which are convergence of mitochondria, endoplasm reticulum and other organelles (NTP, 2014; Allison et al., 2022). In this study. mild and moderate hepatocyteshydropic degeneration was seen in plates C and D respectively. The hepatocytes were swelling and rounding up with watery oedematous wispy rarified cytoplasm that is less eosinophilic than the normal cells. This is consistent with studies of rats exposed to xenobiotics (NTP, 2012; NTP, 2014)

4.2.3. Ballooning Degeneration

Is a severe form of hydropic degeneration. It results in depletion of adenosine triphosphate (ATP) and rise in intracellular calcium, leading to loss of plasma membrane volume control and disruption of the hepatocyte intermediate filament network (NTP, 2014; Allison et al., 2022). Microscopically it can be identified at low power as hepatocytes 2 - 3 times the size of normal adjacent hepatocytes. Ballooned cells lose their usual polygonal shape and become rounded with watery oedematous wispy rarified cytoplasm that is less eosinophilic than the neighboring cells with vacuolization and lacking fat accumulation (NTP, 2014; Allison et al., 2022). These cells usually demonstrate clumping of intermediate

filaments, characterized as strands of eosinophilic, ropey cytoplasmic material referred to as Mallory bodies. In this study, ballooning degermation was seen in plates C and D. It is characterized by their cytoplasm appearing swollen and granular, which tends to condense around the nucleus. This was consistent with toxicological studies carried out by NTP, 2014.

4.2.4. Sinusoidal dilation (SD)

Hepatic sinusoidal dilation refers to the enlargement of the hepatic artery. It usually occurs in in centrilobular area, or less frequently a periportal. Three pathomechanisms have been proposed (NTP, 2014; Allison et al., 2022):

Atrophy of the hepatic cords as a primary event.

Hemodynamic factors remodeling the sinusoidal lumen: Increased hepatic arterial blood flow (e.g. arteriovenous malformations) could cause dilation of sinusoids, relevant in portal vein blood flow deprivation. Localized obstruction of sinusoids may also induce upstream SD, relevant in perisinusoidal fibrosis, nodular regenerative hyperplasia, or SOS (oxaliplatin-associated SD).

Implication of soluble systemic factors including IL-6 and vascular endothelial growth factor (VEGF), relevant in SIRS-associated SD. Activation of neurogenic locus notch homolog protein 1 (Notch 1) pathway (signaling that helps determine the specialization of cells into certain cell types that perform particular functions in the body – cell fate determination) in sinusoidal endothelial cells could also induce SD (Marzano et al., 2015).

In this study, the sinusoidal dilation was diffuse and mainly around the periportal areas. This was consistent with the histological findings in the liver of a xenobiotic exposed rat (Marzano et al., 2015)

4.2.5. Other observed histological alterations

Coagulative necrosis (or acidophilic degeneration) also occurred in this study. It is characterized by the cytoplasm becoming intensely eosinophilic, the nuclei are small and pyknotic and with an eventual extrusion from the cell, leaving behind necrotic, acidophilic mass called councilman body or acidophil body. Lesions also consist of another type of hepatocellular necrosis known as Dropout Ballooning (DB) necrosis in which isolated or small clusters of hepatocytes undergo lysis. There is also Inflammatory Response (IR), which is infiltration by mononuclear inflammatory cells, usually in the portal tracts, but may permeate into the lobules. Findings in this study were consistent with other studies: Mohapatra et al. (2013) showed that histopathological alterations in the liver tissue of heavy metals treated mice manifested by disruption of hepatocytic plates, disintegration of hepatocytes marked by rupture of cell membrane, cytoplasmic vacuolization and pyknosis of nuclei. El-Refaiy (2013) showed severe hepatic necrosis, fatty changes, degeneration signs and inflammatory cell infiltrations of heavy metals administrated rats. The study concluded that, the histopathological changes of the liver treated with heavy metals might be due to the formation of highly reactive radicals and subsequent lipid peroxidation. The accumulated hydroperoxidase can cause hepatoxicity, which is associated with the peroxidation of membrane phospholipids by lipid hydroperoxidase, the basis of hepatocellular damage (Renugadevi, 2010). Jihen et al. (2008) also supported the aforementioned pathogenesis by suggesting that Cd inhibits protein synthesis and glycogen metabolism in liver of heavy metals contaminated rats. Some other results also showed cellular infiltration and hemorrhagic spots of heavy metals treated rat liver also found in agreement with acute and chronic effects of Cd documented by (Mohapatra et al., 2013). Omar, (2013) has also reported relatively hemorrhage in liver of heavy metal treated Wistar rats. It was suggested to be due to the congestion of the blood vessels and blood sinusoids through which blood has escaped. Moreover, some scholars reported that a considerable number of Kupffer cells are observed in the sinusoid walls after heavy metal treatment of Wistar rats. Proliferation and increased number of Kupffer cells could indicate defense mechanism against heavy metals toxicity (Omar, 2013) and (ElRefaiy, 2013).

5. Conclusion

This study was ecologically relevant. It was able to demonstrate that known contaminants of oil spill sites, if consumed at rates equal to or higher than their oral reference dose for mammalian species can cause liver disorders, even at sub-chronic toxicity period. The study has once more given credence to the use of histology as a biomarker to assess sublethal level of environmental stressors, and in determination and extrapolation of the ecosystem pollution capabilities of the exposure to the studied target chemicals.

Compliance with ethical standards

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Disclosure of conflict of interest

No conflict of interest to be disclosed.

Statement of ethical approval

If studies involve use of animal/human subject, authors must give appropriate statement of ethical approval. If not applicable then mention 'The present research work does not contain any studies performed on animals/humans subjects by any of the authors'.

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